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Volume 1
Number 4

Review

The inositol phospholipids

J. N. Hawthorne

Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15, England

[Received for publication April 22, 1960]

The dried crystals melted with decomposition at about 215°. The substance is probably an organic acid but we have not yet had time to examine it thoroughly." (1) In this modest way inositol seems to have made its first appearance as a lipid constituent. Later in 1930, Anderson and Roberts (2) established the identity of their crystals, obtained from a phospholipid present in avian tubercle bacilli, as *myo*-inositol. The occurrence of inositol in a plant lipid, soybean oil, was first reported in 1939 by Klenk and Sakai (3). Three years later Folch and Woolley (4) described an inositol lipid in brain. The subsequent work of Folch, who coined the name phosphoinositide, stimulated the much wider recent interest in such lipids. Their chemistry has been reviewed by Folch (5) and by Folch and LeBaron (6).

NOMENCLATURE

Several systems for the classification and naming of phosphatides have been put forward (7 to 10). The inositol phosphatides prove difficult to accommodate in some of these. Folch and Sperry (7), for instance, divide the phosphatides into three groups: (1) phosphosphingosides, (2) phosphoglycerides, and (3) phosphoinositides. Most of the inositol phosphatides so far described also contain glycerol and could therefore appear in two of these groups. It is perhaps simpler to make only two subgroups: (*a*) glycerophosphatides (containing glycerol but no sphingosine), and (*b*) phosphosphingolipids (containing sphingosine but no glycerol). Derivatives of sphingosine and the closely related phytosphingosine (11) would be classed together. Inositol lipids also containing glycerol would form a subdivision of group (*a*) called "phosphoinositides," as Folch suggested. The inositol lipid described by Carter *et al.* (11) would belong to group (*b*) since it contains phytosphingosine but no glycerol. If this compound is derived from a more complex lipid containing a "phosphatidyl" residue, the latter's classification would be more difficult according to any scheme.

Like the name phosphoinositide, the names monophosphoinositide and diphosphoinositide for specific compounds are also in general use, but have less to recommend them. The substitution of the name phosphatidylinositol for monophosphoinositide has several advantages. First, it brings the compound into line with the better-known phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, which have related structures. Phosphatidyl then has a specific chemical meaning, derived from phosphatidic acid (di-acyl glycerophosphoric acid), according to Channon and Chibnall (12). Second, use of the name phosphatidylinositol allows more complex lipids, such as Vilkas and Lederer (13) described (phosphatidylinosito-di-D mannoside), to be given a related name indicating structure. Third, it avoids confusion of phosphatidylinositol with other plant monophosphoinositides, which also release inositol monophosphate on hydrolysis but are actually quite different. The name glyceroinositophosphatidic acid for phosphatidylinositol (14) could also lead to confusion.

The name lipositol, used by Woolley (15) for an inositol lipid from soya beans, is now rarely used. Its reintroduction would be undesirable since the product was probably a mixture, and the term could be applied to any lipid containing inositol. As Folch and LeBaron point out (6), insufficient structural evidence is available for the naming of other phosphoinositides. When such evidence is produced, it may be possible to replace diphosphoinositide by a more systematic name.

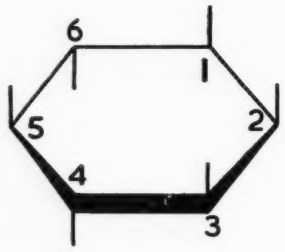
CHEMISTRY OF INOSITOL

Since this subject has been well reviewed by Angyal (16) and Angyal and Anderson (17), only a few points will be mentioned here. There are eight possible isomers of hexahydroxycyclohexane, one of which is racemic, but so far only one has been reported as a lipid constituent. Now called *myo*-inositol (I*a* and I*b*), it was known for many years as *meso- or i*-inositol.

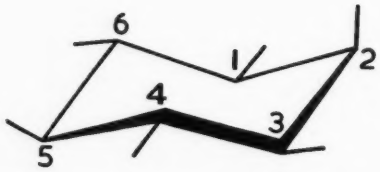
For polyhydroxy compounds, the inositols are surprisingly stable. *Myo*-inositol has been isolated from a rat carcass which had been digested with 6N sulfuric acid overnight in an autoclave at 17 pounds pressure (18). After neutralization of the digest, treatment with charcoal, and passage through a mixed-bed ion-exchange resin, the inositol could be crystallized directly by adding alcohol. Charalampous and Abrahams (19, 20) used a similarly vigorous procedure for the isolation of C^{14}-labeled inositol from yeast grown on C^{14}-labeled glucose. The washed yeast was refluxed for 12 hours with 22 per cent hydrochloric acid. C^{14}-inositol may also be prepared from plants grown in an atmosphere containing $C^{14}O_2$ (21).

Other methods are available for the production of isotopically labeled *myo*-inositol. Weygand and Schulze (22) obtained it in 2.2 per cent yield from $BaC^{14}O_3$ by the following sequence of reactions:

Though *myo*-inositol is often represented for convenience in structural formulas with a planar carbon ring (I*a*), the preferred conformation is actually the "chair" form of the ring giving one axial and five equatorial hydroxyls (I*b*). The photographs (II and III) of a model in this conformation will give a better

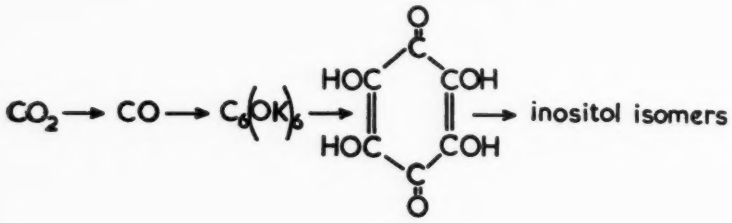


(I*a*)
Myo-inositol, planar carbon ring.



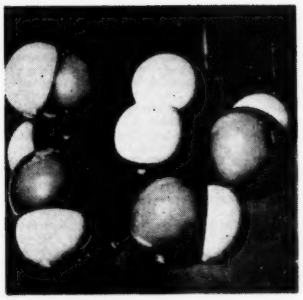
(I*b*)
Myo-inositol, preferred "chair" form.

idea of the spatial relationships. Use of this conformation makes certain reactions easier to understand. For instance, the relatively nonspecific enzyme phytase is able to remove all the phosphate from phytic acid (*myo*-inositol hexaphosphoric acid), but if the reaction is stopped when 70 per cent of this phosphate has been released, inositol 2-phosphate may be isolated from the digest. The phosphate on the axial 2-hydroxyl is less readily hydrolyzed than the other five which have the equatorial configuration. Studies by Ellis (27) have confirmed this. Inositol 2-phosphate was hydrolyzed less readily by prostate phosphomonoesterase than an equatorially substituted phosphate. It is of interest that the natural phosphatidylinositols so far studied have the inositol 1-phosphate structure, avoiding the less reactive 2-position.



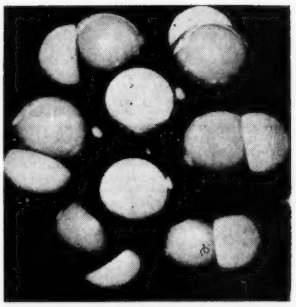
The inositol isomers produced by the catalytic hydrogenation of tetrahydroxybenzoquinone were separated by chromatography on cellulose powder columns in an acetone-water mixture (16). Posternak *et al.* (23) synthesized *myo*-inositol (2-C^{14}) from $C^{14}H_3NO_2$ and aldo-3-0-isopropylidene, 1,2 D xylo-pentofuranose in a seven-stage synthesis. The radiochemical yield was 4.2 per cent. From suitably labeled glucose, inositol labeled in other positions could be synthesized using this method. Wilzbach (24) has described the production of uniformly tritium-labeled inositol by equilibration with tritium gas. Posternak *et al.* (25) produced inositol labeled with deuterium in the 2-position, by the reduction of *scyllo*-inosose. Agranoff *et al.* (26) used a similar method to prepare inositol-2-H^3.

The fascinating studies of Magasanik and coworkers (28, 29) on the oxidation of inositol isomers by *Ace-*



(II)

Model of *myo*-inositol in preferred "chair" form, axial 2-hydroxyl uppermost.



(III)

Same model from above, axial 2-hydroxyl on right.

tobacter suboxydans show that only axial hydroxyls are oxidized to keto-groups. Platinum-catalyzed dehydrogenation (30) gives similar results. This may at first seem to contradict what has been said about the reactivity of axial OH groups. In this case, however, the attack is at the equatorial hydrogen; e.g., *myo*-inositol has only one such hydrogen, in the 2-position, and so the 2-inosose (*scyllo*-inosose) is produced. The conformation of *myo*-inositol is also important in connection with the hydrolysis of diesters based on inositol phosphates. This subject is discussed below.

CHEMISTRY OF THE INOSITOL PHOSPHATES

Courtois (31) has comprehensively reviewed the earlier work on the chemistry and biochemistry of these compounds. Of the different inositol isomers, only *myo*-inositol has been found naturally in combination with phosphate (17). Phytic acid, the best-known phosphoric acid ester of inositol, is dealt with in detail by Courtois and so will be mentioned here only as starting material for the preparation of inositol monophosphate. Posternak and Posternak (32) first carried out this preparation using the phytase of bran, although Anderson (33) had previously obtained the monophosphate by acid hydrolysis of phytic acid. At present the method used most often is the modification of the enzymic method due to McCormick and Carter (34). Fleury *et al.* (35) compared the inositol monophosphates produced by enzymic and alkaline hydrolyses of phytate. Only the former was believed to be identical with synthetic inositol 2-phosphate. More recent work (36, 37) has shown that all three compounds are identical, as would be expected on theoretical grounds (38). This 2-phosphate has less activity as a growth factor for cells in tissue culture than the phosphate obtained by hydrolysis of phosphatidylinositol, which is now known to be a mixture of inositol 1- and 2-phosphates (39).

The esters obtained by hydrolysis of phytic acid have been partly separated on ion-exchange columns (40), and also by paper ionophoresis (41). The behavior on Nalcite SAR columns of inositol monophosphate isolated from liver has been described by Hübscher and Hawthorne (42). Desjobert and Petek (43) were able to separate inositol mono-, di-, tri-, tetra-, penta-, and hexaphosphates on paper chromatograms, though the penta- and hexaphosphates ran to almost the same positions. The developing reagents of Wade and Morgan (44) were used but the alkali-silver nitrate method of Anet and Reynolds (45) is rather more sensitive.

An inositol monophosphate was synthesized in 1931 by Horiuchl (46), who phosphorylated free *myo*-inositol and thus probably obtained a mixture of isomers containing little of the 2-phosphate. Iselin (47) published the first synthesis of an inositol phosphate of known configuration, taking advantage of the enzymic oxidation of the 2-hydroxyl of *myo*-inositol mentioned above. *Scyllo*-inosose thus produced was acetylated and the product reduced in the presence of a platinum catalyst to 1,3,4,5,6-penta-O-acetyl inositol and a small amount of penta-O-acetyl scyllitol. The mixture was phosphorylated with diphenyl phosphochloridate and the scyllitol derivative separated by fractional crystallization. After removal of the protecting acetyl and phenyl groups, inositol 2-phosphate and scyllitol phosphate were obtained. 1,3,4,5,6-penta-O-acetyl-inositol can be prepared in another way, which avoids the enzymic oxidation. Angyal *et al.* (48) acetylated the acetone derivative of inositol, thus obtaining (±)-3,4,5,6-tetra-O-acetyl-1:2-O-isopropylidene-*myo*-inositol. Mild acid treatment converted this to the (±)-3,4,5,6-tetra-O-acetyl-*myo*-inositol. Davies and Malkin (49) acetylated this compound with 1.25 moles of acetyl chloride and obtained 1,3,4,5,6-penta-O-acetyl-*myo*-inositol. This provides another example of the greater reactivity of equatorial than axial hydroxyls in the inositols.

Kilgour and Ballou (50) have synthesized levo-inositol 3-phosphate. The phosphorylation of the tetra-acetate of *myo*-inositol mentioned above appears to

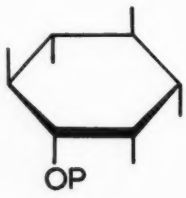
give a cyclic 1:2 phosphate and from this Pizer and Ballou (51) prepared a (±) 1-phosphate by alkaline hydrolysis. Some 2-phosphate is also formed but may be removed since it has a more soluble cyclohexylamine salt.

The *myo*-inositol 2-phosphate obtained from phytic acid may be converted to a 1:2 cyclic phosphate by reaction with dicyclohexyl-carbodi-imide (51, 52). Hydrolysis of the cyclic phosphate in acid or alkali gave a mixture of (±) 1-phosphate (70-80 per cent) and 2-phosphate. These compounds may be distinguished by chromatography on paper in isopropanol: ammonia:water (7:1:2, v/v) and so the above authors have been able to demonstrate phosphate migration for the first time in the inositol series (51, 52, 53). Acid catalyzed the formation of (±) 1-phosphate from 2-phosphate, the final mixture being similar to that produced by hydrolysis of the cyclic *myo*-inositol 1:2-phosphate (53). These results are important in deciding the structure of the naturally occurring phosphoinositides, as will be seen later.

Until recently the inositol monophosphates obtained by hydrolysis of phosphoinositides were believed to be optically inactive, but Pizer and Ballou (51) have now prepared an active 1-phosphate ($[\alpha]_{589}^{25}$ + 3.4° for the cyclohexylamine salt at pH 9) from an alkaline hydrolysate of soybean lipids. Their results have been confirmed by Hawthorne *et al.* (54), who also prepared a 1-phosphate from liver phosphatidylinositol by enzymic hydrolysis. This latter compound appears to be optically active[1] and may be identical with the inositol phosphate which occurs free in liver and other tissues (42).

Brown *et al.* (55) have oxidized glycerylphosphorylinositol prepared from phosphatidylinositol to a glycolaldehyde derivative which broke down to inositol 1-phosphate on treatment with phenylhydrazine at pH 6. There was no evidence of phosphate migration.

Starting from the naturally occurring inositol galactoside "galactinol," first isolated from sugar beet by Brown and Serro (56), Ballou and Pizer (57) have synthesized an optically active *myo*-inositol 1-phosphate. Kabat *et al.* (58) had previously shown that galactinol was 1-O-α D galactopyranosyl *myo*-inositol and that inversion at the inositol-galactose linkage led to (-)-inositol. In the synthesis galactinol is first completely benzylated. After methanolysis to break the galactosidic linkage, the penta-O-benzyl inositol was phosphorylated with diphenyl phosphochloridate. The usual hydrogenation gave an inositol phosphate whose cyclohexylamine salt had $[\alpha]_D$ − 3.2° at pH 9. The soybean inositol phosphate has $[\alpha]_D$ + 3.4°. Ballou and Pizer concluded that the respective structures were:



(IV) Synthetic

(V) Soybean

They proposed to call the soybean compound L-*myo*-inositol 1-phosphate.

Myo-inositol 5-phosphate has also been synthesized (17) but the details are not yet in print.

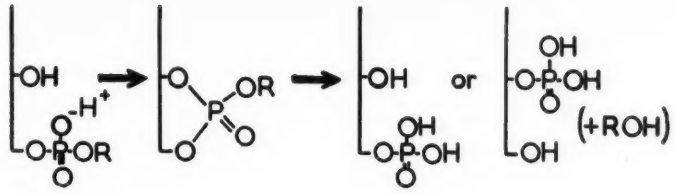
CHEMICAL SYNTHESIS OF PHOSPHATIDYLINOSITOL AND RELATED COMPOUNDS

Davies and Malkin (59) have published the first chemical synthesis of an inositol phospholipid, 2,3-distearyl-glyceryl 2-*myo*-inosityl phosphate. They reacted glycerol 1-iodide 2,3-distearate with silver 2-(1,3,4,5,6-penta-O-acetyl) *myo*-inosityl phenyl phosphate. Protecting groups were removed by hydrogenation followed by reflux with ethanolic hydrazine. This excellent synthesis should stimulate progress in the field of inositol phosphatide biochemistry, where work is being done at present on poorly characterized compounds. Combining this method with that of Ballou and Pizer (57), it should be possible also to synthesize a lipid having the inositol 1-phosphate structure.

Work in two laboratories has led to the synthesis of glycerol 1-(inositol 2-phosphate) by different routes and the work has been published simultaneously (49, 60). In the first method 1,3,4,5,6-penta-O-acetyl *myo*-inositol was treated with 1.1 moles phenyl phosphochloridate in lutidine, followed by the addition of 5 moles (DL)1,2-isopropylidene-glycerol. A crystalline product was isolated which gave glycerol 1-(inositol 2-phosphate) after removal of protecting groups. It is interesting that when D-1,2-isopropylidene glycerol was used, the final product was optically inactive, even though the intermediates were active. In the second method (60) 1,3,4,5,6-penta-O-acetyl *myo*-inositol 2-phosphoric acid was reacted with DL-1,2-isopropylidene glycerol in the presence of dicyclohexylcarbodiimide and pyridine. Ellis and Hawthorne[2] have also prepared

[1] P. Kemp, unpublished observations.

[2] Unpublished results.



(A)

the corresponding scyllitol diester by this second route. Brown *et al.* (55) have synthesized glycerol 1-(inositol 2-phosphate) and glycerol 1-(inositol 1-phosphate) by letting glycidol react with the corresponding inositol phosphates. Their products contained small amounts of the glycerol 2-derivatives.

Glycerylphosphorylinositol has also been prepared by mild alkaline hydolysis of liver phosphatidylinositol (61). The pure diester was isolated by chromatography on Dowex 1 in the presence of borate. It is now known that this compound has the glycerol 1-(inositol 1-phosphate) structure (51, 55, 62).

HYDROLYSIS OF GLYCERYLPHOSPHORYLINOSITOL AND RELATED COMPOUNDS

Though phosphate migration occurs less readily in the inositol phosphates than in glycerophosphate derivates, it can easily invalidate structural conclusions based on the properties of phospholipid hydrolysis products. This was not realized by the earlier workers in the field, but the studies of Brown and his colleagues in Cambridge, using model compounds (37, 55, 63), have considerably improved our understanding of the factors involved. Modern views are based on the theory that an intermediate cyclic triester is formed during the hydrolysis of phosphoric acid diesters having a free hydroxyl vicinal to the phosphate group (Scheme A). The cyclic triester then rapidly breaks down to release the group ROH and a cyclic diester which hydrolyzes to a mixture of isomeric α and β phosphates. The case of phosphatidylinositol[3] is more complicated than this, since once the fatty acids are removed, cyclization is possible with both an inositol and a glycerol hydroxyl. Alkaline hydrolysis could take place according to either Scheme B or Scheme C.

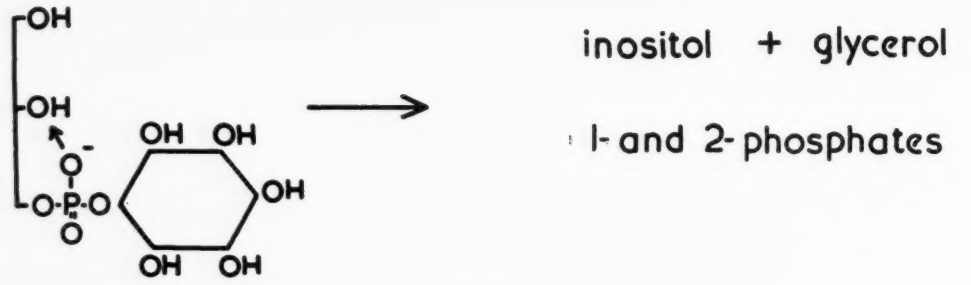
[3] The convention in which the inositol ring is considered planar will be used in this discussion, hydroxyls being described as being in *cis* or *trans* relationship. Use of the nonplanar convention does not affect the argument (16), though the presentation becomes more complicated.

Brown *et al.* (63) studied the corresponding derivatives of *cis*- and *trans*-cyclohexanediol rather than inositol. Alkaline hydrolysis of glycerol 1-(*cis*-2-hydroxycyclohexyl phosphate) gave only 14 per cent glycerophosphate. The remaining phosphate was in the form of 2-hydroxycyclohexyl phosphate, no inorganic phosphate being produced. The *trans* diester gave 77 per cent glycerophosphate. It might be expected that inositol 1(3)- or 2-derivatives would resemble the *cis* compounds, and inositol 4(6)- or 5-derivatives the *trans* (see I*a*). In fact, the situation is complicated by the presence of the other inositol hydroxyl groups. Table 1 summarizes the results of hydrolysis studies on the inositol diesters. Though Brown *et al.* (55) were able to detect a small difference between the inositol 1- and 2-phosphate derivatives, this appears comparable with the experimental error in the analyses of other workers. It would be unwise to make structural decisions on the basis of this difference.

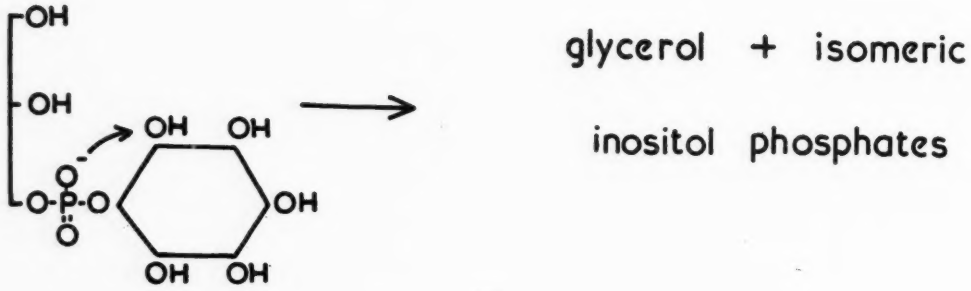
TABLE 1. HYDROLYSIS OF THE GLYCERYLPHOSPHORYLINOSITOLS

Compound	Inositol Phosphate*	Reference
Glycerol 1-(inositol 2-phosphate) *synthetic*	31, 35	(48)
Glycerol 1-(inositol 2-phosphate) *synthetic*	31	(59)
Glycerol 1-(inositol 2-phosphate) *synthetic*	39, 40	(54)
Glycerol 1-(inositol 1-phosphate) *synthetic*	34, 35	(54)
Glycerylphosphorylinositol *from liver*	35	(64)
Glycerylphosphorylinositol *from liver*	39, 34	(65)

* As per cent of total P in the hydrolysate, all of which was organic.

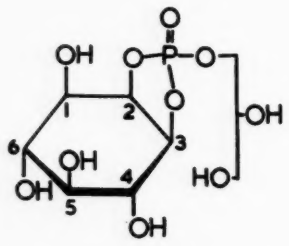


(B)



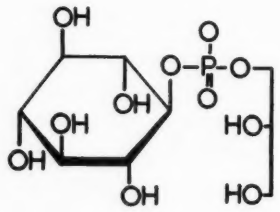
(C)

In all of the synthetic compounds (Table 1) the phosphate has neighboring *cis* hydroxyls on the inositol ring, yet the yield of inositol phosphate is much smaller than in *cis*-cyclohexanediol derivatives. Cyclization is apparently hindered by the presence of a third *cis* hydroxyl in the *myo*-inositol (VI). Evidence from related compounds supports this idea (65).



(VI)

Diesters having the inositol 1(3)- or 2-phosphate structure would be expected to give similar amounts of inositol phosphate on hydrolysis, but inositol 4(6)- or 5-phosphate derivatives would give much less. Glycerol 1-(scyllitol phosphate) (VII), in which the phosphate is flanked by two scyllitol *trans* hydroxyls,



(VII)

should be very similar in its behavior to the inositol 4(6)- or 5-compounds. For this reason it has been synthesized and its hydrolysis studied.[4] Preliminary results indicate that on alkaline hydrolysis only about 3 per cent scyllitol phosphate is produced. This provides further evidence that the natural inositol diester and therefore liver phosphatidylinositol also have the 1(3)- or 2-structure.

[4] R. B. Ellis and J. N. Hawthorne, unpublished observations.

ISOLATION OF PHOSPHOINOSITIDES FROM TISSUES

One of the great obstacles in the study of phospholipids is the lack of easy and reliable methods for the isolation of pure compounds from tissue extracts. Apart from contamination of one phospholipid with another and with nonlipid impurities, the range of fatty acids present in any particular phospholipid makes further difficulties. It is almost impossible to be certain, for example, that phosphatidylinositol isolated from heart muscle has a fatty acid composition identical with that in the original tissue. Suppose, as an illustration, that heart contains only a distearyl and a dioleyl phosphatidylinositol. They will have very different solubility properties. During purification it is most unlikely that identical proportions of each will be lost. If silicic acid chromatography is employed in the isolation, the unsaturated phosphoinositide will appear in the early fractions and the saturated compound may overlap with a phospholipid eluted later from the column. If precipitation with organic solvents is used, there is a possibility that more unsaturated phosphoinositides will be lost in the supernatant. A comparison of the fatty acid composition of phosphatidylinositols isolated by various methods is given in a later section.

Considerable purification of phosphoinositides can be obtained by solvent treatment and reprecipitation. Folch has perfected methods of this type (5, 6), dissolving the lipid in chloroform and using carefully controlled volumes of methanol or ethanol for precipitation. The purity of the product must be checked by analysis, e.g., the nitrogen content will indicate contamination of many phosphoinositides, which are nitrogen-free when pure. The inositol to P molar ratio, which should be a simple one, is also a useful guide.

McKibbin and Taylor (66) developed a method for the fractionation of phospholipids by absorption on silicic acid columns and elution with increasing concentrations of methanol in chloroform. The procedure of Hanahan *et al.* (67) seems to this writer to be the best modification so far described for the preparation of phosphoinositides. These authors used 20 per cent (v/v) methanol in chloroform as their first eluting agent. This removed phosphatidylserine and phosphatidylethanolamine as one peak. The second solvent, 40 per cent methanol in chloroform, removed phosphatidylinositol and then lecithin. The method gave good results with liver phospholipids but has not been applied to those from brain.

Dils (68) has shown that ox brain diphosphoinositide is not separable from phosphatidylserine on silicic acid columns. Vilkas and Lederer (13) have successfully applied silicic acid chromatography to the separation of tubercle bacillus phosphoinositides. Dils and Hawthorne (69) used cellulose acetate columns for the purification of liver phosphatidylinositol. Using countercurrent extraction, Hörhammer *et al.* (70) have separated phosphatidylinositol and diphosphoinositide from an ox brain phospholipid fraction ("Folch fraction I"). These workers also obtained three inositol lipids from soybean fat by the same method.

Although the pursuit of purity is essential for work on the chemistry of phosphoinositides, it takes us a long way from conditions inside the cell, where phospholipids are certainly impure. For biochemical studies it is necessary to isolate larger units as well. Most of the lipoproteins are appalling mixtures from the chemist's point of view, but they are more significant metabolically than their "purified" lipid components. Though different criteria of purity have to be applied, these large units may also be obtained in a "pure" state.

ANALYSIS OF PHOSPHOLIPID MIXTURES

As yet there is no satisfactory method of separating all the individual phospholipids of a tissue from one another. Some fractionation is possible on silicic acid columns or silica-impregnated paper, but neither method can be applied directly to the quantitative analysis of phospholipid mixtures. It seems doubtful if physical methods of this sort will provide a complete separation, since each phospholipid is really a family of molecules with differing fatty acid composition. As already mentioned, the fatty acids may profoundly alter the physical properties of a phospholipid.

Much analytical work on tissue phospholipids has therefore been done in other ways. One method involves a vigorous hydrolysis of the lipid mixture and estimation of individual components such as choline or inositol in the hydrolysate (71, 72, 73). It has the disadvantage that the same component may occur in more than one phospholipid. Another method is based on mild alkaline hydrolysis, which removes only the fatty acids, followed by separation of the resulting phosphate esters (e.g., glycerylphosphorylcholine) by two-dimensional paper chromatography (74) or on ion-exchange columns (61).

These methods, however, provide no help in studies involving the fatty acids of phospholipids, so several workers have turned to paper chromatography of the intact compounds. Lea *et al.* (75) described the separation of certain phospholipids on silica-impregnated

filter paper, but phosphoinositides were not included. Rouser *et al.* (76) used a variety of polar or ionic solvents on untreated filter paper, detecting the phospholipid spots with Rhodamine B or G. Unfortunately, phosphatidylinositol does not move in these solvents (77). Marinetti and Stotz (78) also separated a series of phospholipids on silica-impregnated papers using both one- and two-dimensional chromatography. The method has been applied to phospholipid mixtures from several different tissues (79, 80). Phosphatidylinositol has an R_f of 0.20 in one of the solvents used (78), a mixture of diisobutylketone, acetic acid, and water (40:30:7, v/v). In the same solvent, diphosphoinositide does not appear to move (81). Agranoff *et al.* (26), using this solvent with silica-impregnated glass fiber papers, found that phosphatidylinositol "tailed" from R_f 0.55 back to the origin. They obtained better results without the silica impregnation and in a solvent consisting of diisobutylketone and acetic acid (30:5, v/v). It will be apparent that phosphoinositides do not behave well on paper chromatograms.

Hörhammer *et al.* (82) obtained what seem to be better separations on formaldehyde-treated paper with an *n*-butanol-acetic acid-water solvent (4:1:5). The R_f value of phosphatidylinositol was 0.43. Diphosphoinositide separated into three components (R_f values 0.19, 0.24, and 0.28).

An unusual method for separating phospholipids is paper electrophoresis. This has been used by Zipper and Glantz (83), who separated lecithin, phosphatidylethanolamine, phosphatidylserine, and diphosphoinositide in several different solvents. Relative rates of migration in a solvent consisting of methanol, pyridine, and acetic acid (8:1:1, v/v) made to 0.05 N with sodium acetate, were as follows: Lecithin + (i.e., toward anode) 0.43; phosphatidylethanolamine + 4.12; phosphatidylserine + 150; diphosphoinositide — 27.8. It is surprising that at both acid and alkaline pH, diphosphoinositide moved toward the cathode, while the less acidic phosphatidylserine moved in the opposite direction. Dimyristyl and dioleyl lecithins were not separable. There do not seem to be any other reports of work based on electrophoresis, which, theoretically at least, seems well suited to the separation of phosphoinositides.

ESTIMATION OF LIPID-BOUND INOSITOL

The lack of a simple specific chemical method for the estimation of *myo*-inositol has long been a difficulty in work on the inositol lipids. Microbiological assay with an inositol-requiring yeast has been widely used since Woolley first proposed it in 1941 (84). A reliable and sensitive modern procedure using a strain of *Schizosaccharomyces pombe* is described by Norris and Darbre (85).

Several enzymic methods have recently been proposed. Two of these are based on the oxidation of *myo*-inositol to glucuronic acid by a kidney enzyme (86). Another enzymic assay makes use of an inositol dehydrogenase from *Aerobacter aerogenes* (87). The enzymic and microbiological methods are much more specific than the chemical ones described below, but they involve specialized techniques such as culture of microorganisms and enzyme purification which may not always be available. In addition, inhibitors produced by hydrolysis of a lipid sample may cause difficulty.

The chemical methods that seem most useful are both based on oxidation of *myo*-inositol by periodic acid. Here the chief problem is the removal of interfering compounds such as glycerol, serine, ethanolamine, and the sugars. One version uses paper chromatography to accomplish this and was designed specifically for the analysis of lipid inositol (88). Sealed-tube hydrolysis with a small volume of HCl is employed and the hydrolysate applied directly to paper chromatograms. The chromatographic solvent is isopropanol-acetic acid-water (3:1:1, v/v). Position of the inositol spots is determined by spraying a "marker" strip to which pure inositol is applied. The spots are then cut out and extracted. The extracts are oxidized with periodate and the excess oxidizing agent determined iodometrically. Standards of inositol are put through the whole procedure. The method requires at least 50 to 100 μg. inositol. Hübscher and Hawthorne (42) obtained an accuracy of $\pm$ 3 per cent with it.

A more sensitive method depends on the spectrophotometric estimation in U.V. light of periodate (89). Inositol is oxidized at 65°C under controlled conditions. LeBaron *et al.* (90), as well as Agranoff *et al.* (26), describe this method.

Using the yeast bioassay, Taylor and McKibbin (73) reported the values in Table 2 for the lipid inositol content of various animal tissues. Inositol was released by hydrolysis with 4N HCl under reflux for 40 hours.

PHOSPHOINOSITIDES OF ANIMAL TISSUES

Though Thudichum, the great pioneer of phospholipid chemistry, recognized inositol as a constituent of brain (91), it was not until 1942 that Folch and Woolley (4) found it combined in a brain lipid.

TABLE 2. LIPID-BOUND INOSITOL OF ANIMAL TISSUES*

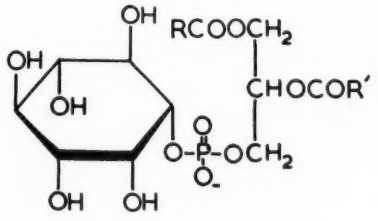
Tissue	Total Lipid Inositol† (μM)	Molar Ratio-Lipid Inositol:P
Dog liver (2)	13.0	.0730
Dog kidney (2)	13.2	.0653
Dog heart (2)	7.39	.0438
Beef heart (1)	8.53	.0546
Dog brain (2)	22.4	.0338
Dog intestine (2)	9.64	.0713
Dog skeletal muscle (1)	3.60	.0480
Dog pancreas (1)	19.3	.0860
Dog lung (1)	10.4	.0500
Dog plasma (2)	6.38	.0253
Rabbit plasma (1)	3.70	.0327
Human plasma (1)	5.05	.0204
Human plasma (10 patients)	7.45	.0255
Human plasma (10 patients) range	5.23-10.7	.0230-.0277

* Reproduced by kind permission of the authors, Taylor and McKibbin (73), and the editors of the *Journal of Biological Chemistry*.

† μM inositol per g. dry lipid-free tissue residue and per 100 ml. plasma. Number of individuals in parenthesis.

Burmaster (92) also showed the presence of inositol phospholipids in brain cephalin. Macpherson and Lucas (93, 94) first showed that liver phospholipids contained inositol.

At present two well-defined phosphoinositides are known in animal tissues, phosphatidylinositol (VIII), whose structure is almost certainly the one shown here, and "diphosphoinositide," isolated by Folch from brain (95, 96).



(VIII)

Hörhammer *et al.* (70) have provided some evidence of a lysophosphatidylinositol in ox brain. Hokin and Hokin (97) obtained two phosphoinositide spots on autoradiograms of pigeon pancreas phospholipids. One appears to be due to phosphatidylinositol, and both give inositol monophosphate and glycerophosphate on hydrolysis. The evidence is not contrary to the idea that the second lipid is a lyso-compound, though the two differ considerably in their turnover of glycerol, inositol, and phosphate.

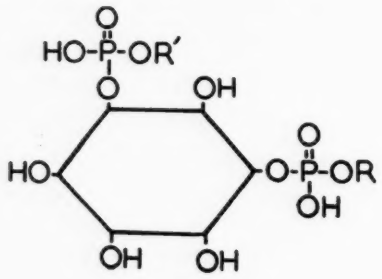
Phosphatidylinositol was first isolated by Faure and Morelec-Coulon from wheat germ (98) and heart muscle (99). McKibbin (100) and Hawthorne (101) obtained evidence of a similar compound in liver. Subsequent work in several laboratories has provided a sound basis for structure (VIII). Faure and Morelec-Coulon (102), after a series of carefully controlled solvent extractions and precipitations, were able to obtain from heart muscle a crystalline sodium salt of phosphatidylinositol. This lipid, therefore, can be added to the very short list of phospholipids which have been obtained from natural sources in crystalline form. The same authors (103, 104, 105), by analysis of the phosphoinositide and its hydrolysis products, showed that it contained fatty acids, glycerol, *myo*-inositol, and phosphoric acid in molar proportions of 2:1:1:1. Analysis of the barium salt of the original lipid showed that the phosphate residue had only one free acid group. Pure preparations were free from nitrogen. The compound from ox heart contained equimolar amounts of stearic and unsaturated fatty acids, the latter having an iodine number of 222 and an average molecular weight of 291. On hydrolysis, inositol monophosphate and glycerophosphate were liberated (106), suggesting that the inositol and glycerol were both linked to phosphoric acid. Confirmation of this was obtained by the liberation of the diester glycerylphosphorylinositol after mild alkaline hydrolysis (107). This diester was isolated and analyzed with ion-exchange columns in the presence of borate (61).

McKibbin's phosphatidylinositol (106), obtained from horse and dog liver by chromatography on silicic acid and countercurrent extraction, contained fatty acids of a much lower iodine number than those of the heart compound (102). The difference may reflect different methods of preparation (108), as has been mentioned before, though a process depending on precipitation, like that of Faure and Morelec-Coulon, would be expected to give more saturated compounds, since these are less soluble. However, these authors point out that their mother liquors did not contain any less saturated phospholipids (108). In addition, silicic acid columns appear to release unsaturated phospholipids quite readily (68, 109), the early fractions of any peak always being highly unsaturated. It is more likely that the difference is due to McKibbin's use of countercurrent extraction, since Hanahan and Olley (64) obtained figures very similar to those of the

French workers. Table 3 summarizes the results on fatty acid composition.

Hanahan and Olley (64) showed that a short HCl hydrolysis preferentially released inositol monophosphate and diglyceride from phosphatidylinositol. Le Cocq and co-workers (110, 111), using acetic acid, also studied this type of hydrolysis. They found that the first product was an optically active cyclic inositol phosphate which then broke down to a mixture of inositol 1- and 2-phosphates. The optical activity and the fact that a cyclic ester could form strongly suggest that the inositol 1-structure was present in the original phosphatide. This inositol 1-structure was first suggested by Pizer and Ballou (36) for soybean phosphatidylinositol, though these authors mention similar findings with the liver compound. Studies of the hydrolysis of glycerylphosphorylinositol from the liver lipid confirm this (64, 112). Brockerhoff and Hanahan (62) also provide evidence that the inositol is esterified in the 1- (or 4-) position. Periodate oxidation of glycerylphosphorylinositol from the liver lipid gave an optically active glycolaldehydeinositol phosphate, which also showed that the glycerol was α-linked. Brown *et al.* (55) used periodate in the same way, as mentioned above, finally obtaining the free inositol 1-phosphate. Their reaction conditions provide an elegant chemical method for the preparation of the natural inositol 1-phosphate.

Diphosphoinositide was isolated from ox brain by Folch (96), who suggested structure (IX) for this lipid.

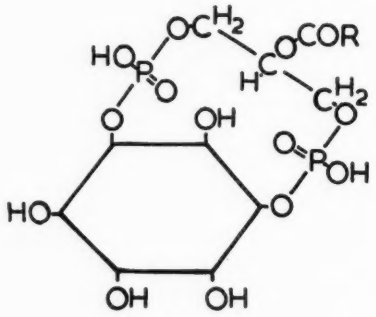


(IX)

(Configuration of ring hydroxyls unknown.)

After many reprecipitations, Folch obtained a compound which appeared to be almost pure. Analysis of its recognized hydrolysis products suggested that all of the compound was accounted for. Inositol, fatty acid, glycerol, and phosphoric acid were found in molar ratios of 1:1:1:2, respectively. When cations were removed, diphosphoinositide had two free acid groups.

On the basis of these analyses and information from periodate oxidation, it was suggested that diphosphoinositide may have the structure (X) (38) or be a polymer in which inositol diphosphate residues were linked by monoglyceride. The latter idea was also put forward independently by Folch and LeBaron (6).



(X)

Recent studies in this laboratory[5] seem to exclude structure (X) and make it even more difficult to envisage the true structure. Carboxylic ester determinations on purified diphosphoinositide from ox brain gave a molar ratio of unity for P:ester. In the original work of Folch (96), the value for this ratio was obtained by weighing the fatty acids from a known amount of lipid and using the equivalent weight of 400 obtained by titration. This figure is unusually high for the equivalent weight of a phospholipid fatty acid. If the more usual figure of 270 is used, Folch's weight data give a molar ratio of 0.87:1 for fatty acid:P. Hawthorne and Chargaff (113) obtained a fatty acid to P ratio of 1.1:1, the equivalent being 330. It seems, therefore, that diphosphoinositide has two fatty acids for each inositol. In the same study phosphatidylinositol was found in brain, confirming the observation of Hörhammer *et al.* (114).

Dittmer and Dawson (115) provide evidence that two triphosphoinositides occur in ox brain. On hydrolysis with acid, inositol triphosphate is released from these complex phospholipids. The simplest composition of one of them is given as $(\text{fatty acid})_6$ $(\text{phosphate})_6$ $(\text{glycerol})_3$ $(\text{inositol})_2$. They are tightly bound to brain protein. Their relation to diphosphoinositide is not yet clear.

The phosphatido-peptides, first described in brain tissue by Folch and LeBaron (116, 117), have attracted the attention of several workers, partly because

[5] J. N. Hawthorne, P. Kemp, and R. B. Ellis, unpublished experiments.

TABLE 3. FATTY ACIDS OF DIFFERENT PHOSPHATIDYLINOSITOL PREPARATIONS

Tissue	Iodine Number	Δ/P*	Equivalent Wt. of Fatty Acids	Nature of Fatty Acids	Reference
Liver	44-56 (fatty acids)	0.42-0.59	236-274	Polyunsaturated + 70% saturated	(106)
Liver	75 (phospholipid)	1.69	287	50% unsaturated 50% stearic	(108)
Heart	78-84 (phospholipid) 222 (unsaturated fatty acids isolated)	1.83	291	50% unsaturated 50% stearic	(103)
Ox liver	———	1.77	250	———	(64)
Rat liver	———	1.90	254	———	(64)

* Double bonds /atom P in the phospholipid.

they contribute phosphate to the RNA fraction in the tissue-P analysis of Schmidt and Thannhauser (118). These substances occur in many tissues and have a high phosphate turnover (119). Phosphatido-peptides seem to be inositol phosphatides linked to peptide or protein by saltlike linkages (117). It is not yet clear whether they occur in this state in the tissues, since the procedures used to isolate them are quite likely to produce artifacts by degradation of the original tissue component. In the fractionation scheme of Schneider (120) phosphatido-peptides appear in the same fraction as phosphoproteins, which also have a high phosphate turnover (121). Huggins and Cohn (119) describe a method for the separation of these two types of compound. A phosphatido-peptide fraction from liver was studied by Hutchison *et al.* (122) and shown to release inositol monophosphate on mild alkaline hydrolysis. A similar fraction from brain contained the diphosphate as well. Glycerophosphate was not observed. Hawthorne (123) obtained evidence that liver phosphatido-peptide contained phosphatidylinositol. These interesting compounds deserve further study from both the chemical and metabolic points of view.

Freinkel (124) showed that sheep thyroid contained a phospholipid releasing inositol and glycerol on hydrolysis.

Spiro and McKibbin (125) have determined the lipid inositol content of various subcellular fractions of liver. Mitochondria, microsomes, nuclei, and supernatant contained 0.07, 0.11, 0.09, and 0.11 moles of lipid inositol, respectively, per atom of phospholipid P. The animals used were rats. A choline-deficient diet did not affect these ratios.

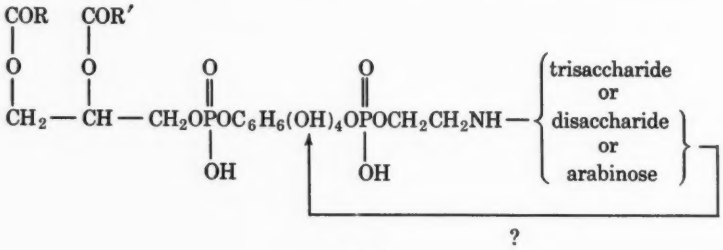
Basford (126) showed that the "co-enzyme Q lipoprotein" from heart mitochondria contained 42 per cent of its phospholipid in the form of lecithin, 48 per cent as "cephalin" (phosphatidylserine plus phosphatidylethanolamine), and 4 per cent as phosphoinositide. This phospholipid pattern differed from that of whole mitochondria as observed by Hanahan—lecithin, 57 per cent; cephalin (as defined above), 28 per cent; phosphoinositide, 9 per cent; sphingomyelin, 1 per cent (see Basford and Green, 127). These authors suggest that other mitochondrial lipoproteins may be richer in inositol lipids and sphingomyelin, each having a specific pattern of components. The role of lipids in electron-transfer systems has been reviewed by Green and Lester (128).

In 1949 Celmer (8) was able to find inositol in the phospholipids of egg yolk. Malangeau (129) studied a phosphoinositide from the same source which had the unusual inositol to P molar ratio of 2:1. Rhodes and Lea (130) and Dils and Hawthorne (131) also reported the occurrence of small amounts of inositol phospholipids in egg yolk.

Lovern and Olley (132) found two types of phosphoinositide in haddock flesh. They concentrated on countercurrent distribution between petroleum ether (b.p. 40°-60°C) and 85 per cent ethanol at the extremities of the system. Garcia *et al.* (133) found a different phosphoinositide pattern when cod flesh lipids were distributed in the same solvent system. The ethanol-soluble compound was the predominant inositol lipid, but one of intermediate solubility was also found. The petroleum ether-soluble component of haddock was absent in cod. Garcia *et al.* also observed that 84 per cent of the cod lipid inositol was retained on a cellulose powder column used for removing water-soluble contaminants. The solvent was chloroform-methanol-water (80:20:2.5, v/v). This retained lipid was eluted by methanol. With haddock, only 19.4 per

cent lipid inositol remained on the column under similar conditions.

An inositol phospholipid occurs in the tapeworm *Taenia saginata* (134). On hydrolysis it released inositol monophosphate. Phospholipid accounted for only 4.3 per cent of the total lipid in this organism. Phosphatidylethanolamine was not found, but lecithin was present. Saha and Igarashi (135) have studied the inositol lipids of certain aquatic animals.



(XI)

VEGETABLE PHOSPHOINOSITIDES

Klenk and Sakai (3) made the first study of a plant inositol lipid by the isolation of inositol monophosphate from soybean phospholipid. They also reported, but did not examine closely, a dextrorotatory phosphate associated with the inositol phosphate. This substance gave a positive Molisch test. Woolley (15) obtained an inositol-containing fraction from the same source, and named it lipositol. On hydrolysis, lipositol released galactose, ethanolamine, fatty acids, inositol monophosphate, and tartaric acid. Folch and LeBaron (6) consider that the tartaric acid was an artefact, possibly due to the vigorous hydrolysis conditions used by Woolley, since Folch (5) was unable to detect this acid in soybean inositol lipid. In the latter study Folch fractionated dialyzed soybean phospholipids by chloroform-ethanol partition until the composition of his product remained constant. At this stage the phosphoinositide contained "hexose" [2], fatty acid [3], primary amine [1], glycerol [1], inositol [2], and phosphoric acid [2]. The figures in brackets represent approximate molar ratios; 97.2 per cent of the weight of material was accounted for by these components. Nomura[6] (136) obtained a similar lipid from soybean "cephalin." On hydrolysis it gave inositol monophosphate, glycerophosphate, galactose, and arabinose.

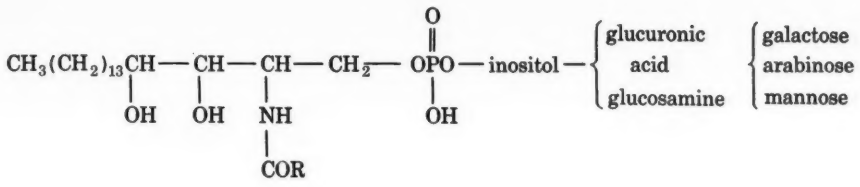
Using peanut (groundnut) lipids as starting material, Hutt *et al.* (138) also found a phospholipid that contained inositol and sugars. Further study of this material led Malkin and Poole (139) to suggest tentatively the following structure (XI). It is probable that Malkin and Poole were dealing wth a mixture of inositol lipids. They found a P:N molar ratio of 1.85:1. Ethanolamine appeared to be the only base. Inositol monophosphate, galactose, arabinose, and a possible disaccharide of galactose and arabinose were found among the hydrolysis products. Glycerophosphate was also found, but no inositol diphosphate.

Scholfield *et al.* (140, 141) fractionated soybean phospholipids by countercurrent distribution in a hexane-95 per cent methanol system. They found two quite different phosphoinositide fractions. Surprisingly, the one containing arabinose, galactose, and mannose in addition to inositol was more soluble in hexane than the other, which contained no sugars. This second compound was more soluble in methanol and nitrogen-free, though contaminated at first with phosphatidylethanolamine (142). It is now known to be phosphatidylinositol.

The most impressive study of seed oil phosphoinositides is that of Carter *et al.* (143). They found phosphatidylinositol and two new types of inositol lipid in most of the oils. The first of these, "lipophytin," contained inositol polyphosphate, fatty acids, some glycerol, and amino acids. Analysis showed 11 to 12 per cent P. The second, "phytoglycolipid," was a complex alkali-stable lipid. In 1953 Van Handel (144) had obtained evidence that soybean contained a lipid of this type. Resistance to dilute alkali provided the key to its purification. Phytoglycolipid was found in corn, flaxseed, soybean, peanut, cottonseed, and sunflower seed. Suitable hydrolyses released the following components: phytosphingosine (145), fatty acids, inositol, glucosamine, hexuronic acid, galactose, arabinose, mannose, and phosphoric acid. The following tentative structure (XII) was put forward (11).

The glucuronic acid and glucosamine were linked as hyalobiuronic acid (146). It was considered that the original inositol lipid might have been a phosphatidyl

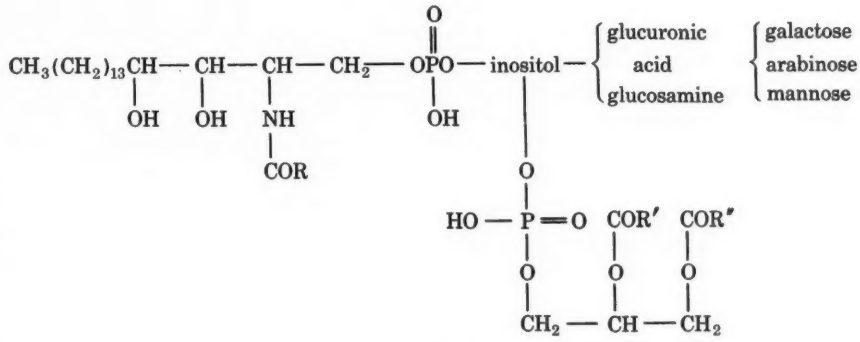
[6] Imai (137) has also studied the phosphoinositides of soybean, but I have been unable to obtain the original publication.



(XII)

derivative of (XII), which gave (XII) on treatment with alkali, since the solubility of phytoglycolipid and the original compound were quite different. Mild alkaline hydrolysis of (XIII), however, would be more likely to remove fatty acids and leave an inositol diphosphate derivative.

phosphatidylinositol was present. This same compound was isolated from wheat germ by Faure and Morelec-Coulon (98, 148). It is also present in peas, where it makes up 5 per cent of the total lipids (149). Peas provide a good starting material for the preparation of pure phosphatidylinositol by a relatively simple



(XIII)

Hawthorne and Chargaff (113), hydrolyzing what was probably a relatively impure soybean phosphoinositide, obtained strongly dextrorotatory compounds (cf. 3) which appeared to be glycosides of inositol monophosphate. They could be separated from inositol monophosphate itself by paper chromatography, or by ion-exchange columns (38). Arabinose and galactose derivatives were present and analysis indicated that each had inositol, sugar, and phosphate in equimolar proportions. The presence of other constituents was not excluded. It is not yet clear whether these substances are breakdown products of phytoglycolipid or another still unknown plant phosphoinositide. Nor is it clear whether Folch's soybean phosphoinositide (5) is a mixture of phytoglycolipid and phosphatidylinositol or whether it contains a third inositol lipid different from both.

Okuhara and Nakayama (147) methylated and degraded a soybean phosphoinositide, concluding that process (150). Hörhammer *et al.* (70), using countercurrent extraction, found three inositol lipids in the alcohol-insoluble fraction of soybean phospholipids. They were phosphatidylinositol, lysophosphatidylinositol, and a more complex sugar-containing phosphoinositide.

McGuire and Earle (151) found 5.7 per cent inositol in crude linseed phospholipids. Countercurrent distribution by the method already mentioned (140, 141) showed that two phosphoinositides were present. The one more soluble in alcohol had a molar ratio of approximately 2:1:1 for P:N:inositol. The corresponding ratio for the hexane-soluble compound was approximately 4:1:1. The authors point out that the compounds were not necessarily pure.

In cottonseed oil, Olcott (152) found a phospholipid resembling lipositol (15).

In rubber latex, Smith (153) found an inositol phospholipid that released reducing sugar on hydrolysis.

The constituents analyzed occurred in the following molar proportions: inositol:sugar:phosphoric acid: fatty acid, 1:1:1:2. The latex lipid contained 10.5 per cent of this substance.

Fuller and Tatum (154) found that most of the inositol in *Neurospora crassa* was bound in phospholipid. The relation between morphology and phosphoinositide content was studied. A colonial form resulting from inositol deficiency contained only 20 per cent as much phosphoinositide as the normal strain.

PHOSPHOINOSITIDES OF MICROORGANISMS

The classical work in this field was done by Anderson and his colleagues at Yale. Without the aid of modern techniques such as chromatography, they made great advances in phospholipid chemistry. In 1939 Anderson (155) reviewed the subject.

After the first discovery of inositol in a phospholipid, which has already been referred to (1), Anderson and Roberts (2) went on to study the inositol lipids of several strains of *Mycobacterium tuberculosis*. From human, avian, and bovine strains, a phospholipid containing inositol and mannose was obtained. Glucose and fructose were also found in the lipid from the human strain. Hydrolysis of the wax from this strain, which differs considerably in solubility from the phospholipid fraction, gave fatty acids and a complex phosphorylated polysaccharide (156). Hydrolysis released arabinose, mannose, and galactose from the latter, but only traces of inositol.

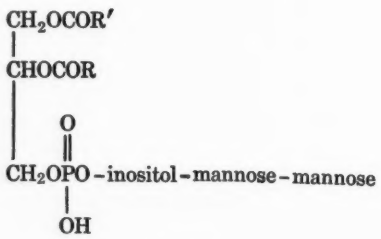
Chargaff *et al.* (157) showed that the nonpathogenic "Timothy grass bacillus" *(Mycobacterium phlei)* contained much less lipid than *M. tuberculosis*. The phospholipid of *M. phlei*, like that of *M. tuberculosis*, had little nitrogen (0.22 per cent). Hydrolysis released 2 per cent inositol, 9 per cent mannose, 10 per cent glycerophosphoric acid, and 60 per cent fatty acids; 19 per cent of the water-insoluble material could not be accounted for (158).

Since all the acid-fast bacteria investigated by Anderson and his collaborators appeared to contain little nitrogenous phospholipid and significant amounts of unusual phosphoinositides, another acid-fast organism was studied, the so-called *Bacillus leprae*[7] (159). This also proved to have a phospholipid which hydrolyzed to a mixture of palmitic acid, oleic acid, high molecular weight liquid fatty acids, glycerophosphoric acid, inositol, mannose, and another sugar. The alcohol-insoluble material obtained by alkaline hydrolysis seemed to be a mixture of glycerophosphate and a phosphorylated polysaccharide (4.7 per cent P). On further hydrolysis with boiling 3.5 per cent sulfuric acid, the polysaccharide was broken down to mannose (2 moles), inositol (1 mole), glucose and fructose (taken together, 1 mole).

In a more detailed study of the human *M. tuberculosis* phospholipid, Anderson *et al.* (160) obtained a polysaccharide by mild alkaline hydrolysis. This, when hydrolyzed with ammonia, lost its phosphate and gave a nonreducing polysaccharide which the authors called "manninositose." It contained 2 moles of mannose for every mole of inositol. Its molecular weight and analysis strongly suggested that it was a dimannoside of inositol. A compound of glycerol, mannose, and phosphoric acid was also thought to be present in the original lipid hydrolysate.

From the same source, de Sütö-Nagy and Anderson (161) isolated several different organic phosphates. The nature of these compounds varied considerably from one culture to the next when synthetic media were used. One of the phosphates was called inositol glycerol diphosphoric acid. It contained no sugar and liberated inositol phosphate and glycerophosphate on hydrolysis. The yields of these compounds are not given, and it is possible that the phosphate was a mixture containing glycerylphosphorylinositol.

Using silicic acid columns to fractionate their lipid mixtures, Vilkas and Lederer (13) have shown the presence of two distinct phospholipids in a streptomycin-resistant mutant of *M. tuberculosis* (H 37 Rv). One of these phospholipids was the magnesium salt of phosphatidic acid and the other was a complex phosphoinositide, phosphatidylinosito-di D-mannoside (XIV), also in the form of its magnesium salt. The fatty acids of these phospholipids were saturated, in



(XIV)

contrast to those found by Anderson. They consisted of palmitic and stearic acids. Vilkas (162) has shown that the mannose residues are linked 1:4 or 1:6 as a mannobiose, which is then linked through its free 1-

[7] Now called *Mycobacterium leprae*.

position to inositol. The point of attachment on the inositol ring is not yet known. In the strain BCG, Vilkas also found evidence of phosphatidylinositol itself, as well as phosphatidylinosito-mono-mannoside, phosphatidylinosito-di-mannoside, and phosphatidylinosito-penta-glucoside.

Cason and Anderson (163) showed that the wax of bovine *M. tuberculosis* contained inositol lipids. The same was true of the human strain wax (164). Nojima *et al.* (165) found 3 per cent inositol in the Wax D fraction of BCG, and in further work Nojima (166) showed that the inositol compounds were phosphatidylinosito-di-, tetra-, and penta-mannosides.

Pangborn (167) has recently isolated serologically active lipopolysaccharides (similar to the compounds above) from pyridine extracts of acetone-dried *M. tuberculosis*. One of the compounds contained 1.73 per cent P, 10.54 per cent inositol, and 50 per cent mannose. The fatty acids were saturated.

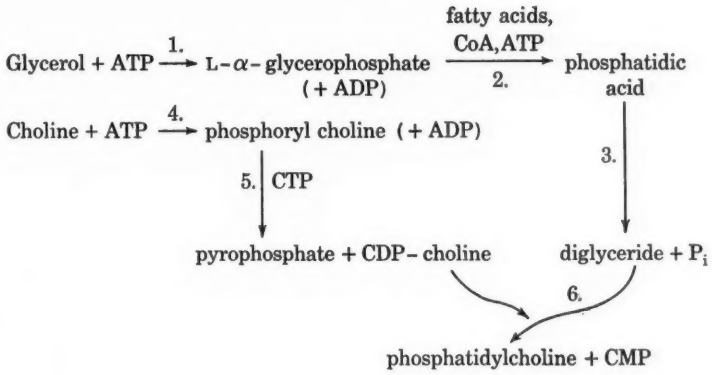
A phosphoinositide fraction has been obtained from yeast by Hanahan and Olley (64). Analytical data and behavior on silicic acid columns suggested that it was phosphatidylinositol. Yarbrough and Clark (168) showed than an inositol-requiring yeast, *Schizosaccharomyces pombe*, incorporated most of its inositol into an alcohol-soluble lipid. No "cephalin-like" phospholipids were detected.

Penicillium chrysogenum contains an inositol lipid which liberates the monophosphate on hydrolysis (169).

BIOSYNTHESIS

In 1939 Chargaff (170) suggested a pathway for phospholipid biosynthesis in which "amino-ethyl alcohol or choline is phosphorylated and the resulting phosphoric acid esters combine with a diglyceride." Recent studies have led Kennedy and his colleagues to the same conclusion. In addition, they have shown for the first time the importance of cytidine nucleotides in phospholipid biosynthesis (171). Kennedy's suggested scheme for phosphatidylcholine or phosphatidylethanolamine is summarized in Scheme D.

Though a full discussion of this scheme is not possible here, one point needs to be made because it bears on the biosynthesis of inositides. The evidence for reaction 4 in animal tissues is not overwhelming. The enzyme catalyzing it, "choline phosphokinase," was isolated from brewer's yeast by Wittenberg and Kornberg (172), but these authors found that liver, brain, and kidney had only one-tenth to one-fortieth the activity of yeast. In addition, Kennedy failed to show the incorporation of free choline into the lipids of isolated liver mitochondria (171). His successful synthetic studies use phosphoryl choline, as do those of several other workers (171, p. 338). However, Berry *et al.* (173) found choline phosphokinase activity in nervous tissue. Dils and Hübscher (174) showed the incorporation of labeled choline into the lecithin of rat liver mitochondria. Their system differed from Kennedy's in that CMP proved a more effective co-



SCHEME D

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; CTP, cytidine triphosphate; CDP-choline, cytidine diphosphate choline; CMP, cytidylic acid; CoA, coenzyme A.

factor than CTP. Preliminary experiments, furthermore, showed that the incorporation of choline or serine was stimulated by calcium ions, alone or in the presence of other cofactors (175). Though it is tempting to think that the calcium-activated incorporation of choline and serine is due to phospholipase action,

(E) phosphatidylcholine $\rightleftharpoons$ choline + phosphatidic acid,

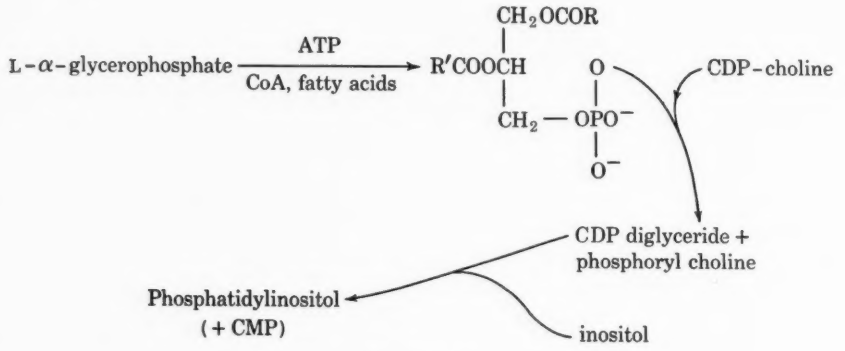
the exact mechanism has not been elucidated.

Work on the biosynthesis of inositol lipids began before the chemical structure of any was established. For these compounds and for phosphatidylserine a pathway similar to Scheme D was sought (176, p. 137). Such a pathway would require inositol monophosphate as an intermediate in the synthesis of phosphatidylinositol. This compound does in fact occur in several tissues (42), but attempts to show the phosphorylation of inositol by tissue extracts in the presence of ATP have been unsuccessful. In a thorough investigation, Hübscher (177) failed to find enzymic activity in any of the following tissues: rat liver, heart, kidney, brain, and intestine; dog liver, kidney, and heart; pig intestine; baker's and brewer's yeast. Two separate assay systems were used. One depended on removal of nucleotides by charcoal and estimation of the increase in non-nucleotide organic phosphate. In the other, ADP formation was measured by addition of phospho-enol-pyruvate and lactic dehydrogenase to give a diphosphopyridine nucleotide-linked system.

Hoffman-Ostenhof *et al.* (178) reported that partially purified preparations of yeast hexokinase were able to phosphorylate inositol in the presence of ATP and Mg^{++} ions. Brain hexokinase also phosphorylates inositol, but at a much slower rate than glucose (179). Hübscher (177) confirmed the yeast hexokinase phosphorylation, but considered it of little significance, since the rate was less than one-sixtieth of that with glucose. It seems more likely, therefore, that the inositol phosphate found free in tissues is a breakdown product of phosphatidylinositol. An enzyme hydrolyzing this lipid, according to reaction (F) below, has been isolated from rat liver (180). It appears to be widely distributed.

(F) phosphatidylinositol + $H_2O \rightarrow$ α,β-diglyceride + inositol monophosphate

Agranoff *et al.* (26) studied the incorporation of tritium-labeled inositol into the lipids of guinea pig kidney and other tissues. Kidney mitochondria, in the presence of Mg^{++} and CMP or CDP-choline, were able to incorporate inositol into a lipid. Chromatography of the lipid and its water-soluble hydrolysis product suggested that it was phosphatidylinositol. Incorporation of labeled inositol was stimulated by phosphatidic acid, but not by D-α,β-diglyceride. In the absence of inositol, kidney preparations were able to catalyze the synthesis of a substance soluble in chloroform-methanol mixtures and believed to contain cytidylic acid. The substance was thought to be CDP-diglyceride. Agranoff *et al.* suggested the following scheme of biosynthesis:



SCHEME G

The scheme differs from that for phosphatidylcholine in that the phospholipid P comes from glycerophosphate. Inositol is incorporated directly into the lipid, so that inositol phosphate does not appear as an intermediate. This would explain the failure of earlier workers to find an "inositol kinase."

The same workers make an interesting suggestion

about the relatively high P^{32}-labeling *in vitro* of phosphatidic acid and inositide in certain tissues (74, 181). Evidence for the presence of phosphatidic acid in animal tissues has been doubtful until the recent isolation of the pure compound from ox liver by Hübscher and Clark (182, 183). Phosphatidic acid phosphatase (184), which hydrolyzes phosphatidic acid to diglyceride and inorganic phosphate, is inhibited by Mg^{++}. If the *in vitro* studies use a higher Mg^{++} concentration than is found *in vivo*, phosphatidic acid will be available for inositide synthesis instead of being hydrolyzed to diglyceride. This cannot be the complete explanation for brain, however, because the high P^{32} incorporation is also observed *in vivo* (185). The great difference in phosphate turnover between phosphatidylcholine and the phosphoinositides in brain does suggest that they are synthesized by different routes. In liver, though, this difference is not found (186).

Paulus and Kennedy (187, 188) consider that there are two possible mechanisms by which inositol may enter lipid molecules. The first is an exchange reaction catalyzed by guinea pig liver microsomes in the presence of Mn^{++} and tris (*tris* [hydroxymethyl] amino-methane), but not phosphate, buffer. Using phosphate buffer and low concentrations of Mn^{++}, the reaction is stimulated by the cytidine derivatives which Agranoff *et al.* quote (26). Otherwise the nucleotides have no effect.

The second mechanism is a true synthesis, stimulated by CTP. The evidence of Paulus and Kennedy (188) supports Agranoff's pathway (G) except that the former workers consider that CTP, rather than CDP-choline, is involved in the formation of CDP-diglyceride. Using a homogenate of whole guinea pig liver, they showed that inositol reduced the yields of an ether-soluble nucleotide, thought to be CDP-diglyceride. This would be expected if CDP-diglyceride reacts with inositol to give phosphatidylinositol and (ether-insoluble) cytidylic acid. Paulus and Kennedy also synthesized CDP-dipalmitin chemically, using dicyclohexylcarbodiimide. Addition of this compound greatly increased the incorporation of tritium-labeled inositol into the lipids of washed, dialyzed chicken liver microsomes. The phosphate of phosphatidylinositol was believed to come from glycerophosphate, in contrast to the results of McMurray *et al.* (189) on brain tissue.

This work illustrates one difficulty in working with radioactive intermediates in subcellular particulate systems. The tracer method is in a way too sensitive, and may detect reactions that have little significance *in vivo*. A false picture can result, for example, when these reactions are the reverse of those taking place in the tissues. The exchange reaction above may be due to an enzyme catalyzing the reaction:

(H) phosphatidylinositol $\rightleftharpoons$ phosphatidic acid + inositol,

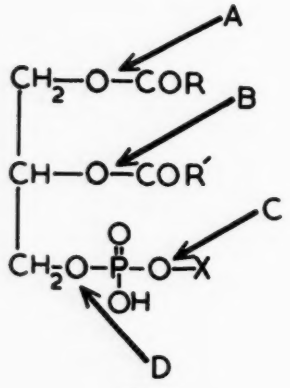
though there is no other evidence for such a phospholipase. At equilibrium there may be 99 per cent hydrolysis of phosphatidylinositol, yet labeled inositol would appear to show synthesis of inositide because the reaction is reversible. Dils and Hübscher make the same point in their work on Ca^{++}-activated incorporation of choline into lecithin (174). In tracer studies, supporting chemical evidence of net synthesis is desirable.

Some work has been done on the incorporation of labeled compounds into the inositides of brain tissue, but, as McMurray *et al.* (189) point out, speculation about biosynthetic pathways is unprofitable until we know the structure of diphosphoinositide. Rossiter and his colleagues have studied uptake of P^{32} into rat brain phospholipids, using two systems, a hypotonic anaerobic system supporting labeling under optimal conditions for glycolysis and an aerobic system requiring conditions for oxidative phosphorylation. Thompson *et al.* (190) found CTP and CDP more effective than CMP or CDP-choline in stimulating incorporation of tritium-labeled inositol into the lipids of such systems. Phosphatidic acid increased labeling in the glycolyzing homogenate only. McMurray *et al.* (189) found that CTP stimulated the incorporation of P_i^{32}, but not P^{32}-labeled glycerophosphate into brain inositide. The biosynthetic problem in nervous tissue is further complicated by the recent demonstrations that phosphatidylinositol occurs in brain tissue as well as diphosphoinositide (81, 70). In addition, Hokin and Hokin showed that the former lipid had a much higher turnover of phosphate and inositol than diphosphoinositide. These authors also showed that acetylcholine stimulates incorporation of labeled phosphate and inositol equally into the phosphatidylinositol of brain slices. Inositol phosphate appeared to be incorporated as a unit into the inositide, while C^{14}-labeled glycerol incorporation was not stimulated by acetylcholine. These observations are not easy to reconcile with the pathway suggested by Agranoff.

ENZYMES HYDROLYZING PHOSPHOINOSITIDES

The phospholipases, of which the best known are the lecithinases, may be divided into four groups,

A, B, C, and D, each group attacking a different bond in a phospholipid of the structure shown (see review on lecithinases by Hanahan [191]).



(XV)

The B enzyme is more properly a lysophospholipase B.

The pathway by which phospholipids such as lecithin are broken down in animal tissues is far from clear (72), but there is less information still about the phosphoinositides.

Long and Penny (192) found that moccasin venom phospholipase A had no action on diphosphoinositide or phosphatidylinositol. Sloane-Stanley (193) first reported the enzymic hydrolysis of an inositol lipid. He used diphosphoinositide as substrate and worked with brain homogenates. Rodnight (194) showed that the enzyme occurred in other tissues such as liver, kidney, and spleen, and that it was activated by Ca^{++}. Inorganic phosphate and an organic ester believed to be inositol monophosphate were released from diphosphoinositide. Kemp *et al.* (195, 196) obtained the same type of enzyme from liver in a purer state. Phosphatidylinositol was also hydrolyzed by the enzyme. None of the other phospholipids studied, which included lecithin and cardiolipin, were attacked. Inorganic phosphate was not produced, and the authors considered that its presence in their less pure enzyme preparations indicated contamination with a phosphomonoesterase. Pileggi (197) has shown the presence of "phytase" activity in several animal tissues. Liver extracts were also able to hydrolyze inositol monophosphate.[8] The "phosphoinositidase" appeared to break down phosphatidylinositol to diglyceride and inositol 1-phosphate, but the products of its action on diphosphoinositide remain obscure.

[8] P. Kemp, unpublished observations.

Phosphatidylinositol has the ability to activate certain phospholipases. Dawson (198) showed that in concentrations as low as 30 μM it had this effect on a phospholipase B from *Penicillium notatum*. Lecithin was used as substrate. Liver "polyglycerophosphatide" (199) had similar activating properties. The *P. notatum* enzyme preparation was able to hydrolyze phosphatidylinositol itself, giving a mixture of glycerylphosphorylinositol, free inositol, inorganic phosphate, and fatty acids (200). This breakdown was inhibited by lecithin or lysolecithin. In contrast, the liver enzyme was not affected by these lipids (195, 196). Pancreatic phospholipase was also able to hydrolyze phosphatidylinositol. In this case diglyceride and inositol monophosphate may have been formed, but the evidence was not conclusive.

The mechanism of attack on lecithin by the *P. notatum* enzyme has been studied by Bangham and Dawson (201, 202). For enzymic attack to take place, they showed that the lecithin micelles had to have a net negative charge. This could be imparted by a variety of substances including dodecyl sulfate and dicetyl phosphoric acid, as well as the lipids cited above. Positive ions, such as Ca^{++}, inactivated the system. The nature of the net charge was shown by electrophoresis. An elegant and novel method of assaying phospholipase activity was used in some of these experiments. A film of P^{32}-labeled lecithin was spread on a Langmuir trough and a Geiger counter arranged above it. Hydrolysis of the lecithin released phosphate into the underlying aqueous phase, so reducing the rate of counting.

The lecithinase C of cabbage[9], which released choline from the lipid, is also activated by phosphatidylinositol (203). Maximum activity was obtained in the presence of Ca^{++}. In the case of the *P. notatum* enzyme, Ca^{++} appeared to inhibit activation by phosphatidylinositol.

PHOSPHOINOSITIDE TURNOVER STUDIES

Dawson (74, 204) showed that the incorporation of P_i^{32} into brain phosphoinositide and phosphatidic acid was greater *in vitro* than that into the other phospholipids. Brain dispersions were incubated with P_i^{32} in the presence of suitable cofactors. The hydrolysis products of the labeled phospholipids were separated by two-dimensional paper chromatography. It is doubtful whether the phosphate esters from phosphatidylinositol and diphosphoinositide separate well under these conditions. When these experiments and the

[9] Unfortunately, sometimes known as lecithinase D.

similar ones of Hokin and Hokin (181) were performed, only diphosphoinositide was thought to occur in brain. Hokin and Hokin (81), in a more recent paper, state that in brain slices similarly incubated, phosphatidylinositol rather than diphosphoinositide was the heavily labeled compound. Results were based on chromatography of the intact phospholipids and electrophoresis of their hydrolysis products.

McMurray *et al.* (189) confirmed Dawson's results in two systems which are described in the section on biosynthesis. Under conditions for anaerobic glycolysis, as well as those for oxidative phosphorylation, inositol phospholipid and phosphatidic acid were much more heavily labeled than the other phospholipids. Identification was again based on paper chromatography.

Similar high turnover of P^{32} in rat brain phosphoinositide was found *in vivo* 3 hours after injection of the isotope into young animals (185). The specific activity of phosphoinositide was about ten times that of phosphatidylcholine. Certain psychotropic drugs affect phospholipid metabolism, though this is not necessarily specific or related to their therapeutic effects. Using brain slices, Magee *et al.* (205) found that Azacyclonol (10^{-3}M) stimulated synthesis of phosphoinositide, phosphatidylserine, and phosphatidic acid, but depressed that of phosphatidylcholine and phosphatidylethanolamine. Higher drug concentrations (5×10^{-3}M) depressed biosynthesis in all cases. Ansell and Morgan (206) found this same depression *in vivo* with rat brain, after a dose of 200 mg. per kg. body weight. Reserpine® (5 to 10 mg. per kg.) had no effect.

In liver, on the other hand, no great difference between the P^{32}-labeling of phosphatidylinositol and the other phospholipids was observed *in vivo* with rats (186). Six hours after injection of the radioisotope, phosphatidylcholine showed the highest P^{32} uptake, whereas phosphatidylethanolamine and phosphatidylinositol had about half the amount of P^{32}. Marinetti *et al.* (79), using rat liver homogenates, also failed to find a high turnover of inositol lipid. High P^{32} uptake was observed only in phospholipids resembling phosphatidic acid, but even this was not seen *in vivo* (76, 78).

Hokin and Hokin found relatively high incorporation of P_i^{32} into pancreas slices (207). Acetylcholine raised the rate of incorporation still farther. The results with pancreas and other tissues are summarized in Table 4.

Freinkel (124) incubated sheep thyroid slices with P_i^{32} or C^{14}-labeled glycerol. After 4 hours the phosphoinositide had almost five times the specific activity of the lecithin, when P^{32} was used. With labeled glycerol there was, if anything, less incorporation into the phosphoinositide than into lecithin, but the rates were low in both cases. This is probably because the glycerol derivatives needed for phospholipid biosynthesis (e.g., diglyceride, glycerophosphate) are available in the tissue slices. Thyrotropic hormone stimulated phosphoinositide-P turnover (see Table 4).

The Hokins have made the general suggestion that phosphoinositides are concerned with the active transport of proteins and other substances out of the cell. Higher turnover of phosphate in phosphoinositide was observed under conditions which caused secretion of amylase, epinephrine, ACTH, mucin, and thyroxin from suitable tissue slices (references in Table 4). Under the same conditions phosphatidic acid turnover was usually stimulated too, and a theory has been put forward that this lipid plays a part in the transport of cations across membranes (213, 214). According to this theory, phosphatidic acid is synthesized from diglyceride and ATP at the inner surface of a membrane consisting essentially of lipid material. This phosphatidic acid diffuses through the membrane, taking with it the cations. At the outer surface of the membrane phosphatidic acid phosphatase hydrolyzes phosphatidic acid, releasing inorganic phosphate and the cations, while the diglyceride diffuses back to the inner surface to be used again. In the case of the albatross salt gland, where sodium chloride rather than sodium phosphate is secreted, an additional mechanism for the removal of phosphate ions would be required. While phosphoinositide may be functioning in some similar way, only further work will lead to an understanding of the increased turnover at the molecular level.

Several interesting points arise from the work of the Hokins, however. When slices were incubated with C^{14}-glycerol instead of P_i^{32}, no increased turnover with acetylcholine was seen. The authors have suggested (81) that the phosphate and inositol of phosphatidylinositol turn over independently of the diglyceride, and that in brain tissue at least, inositol phosphate is incorporated into the phospholipid as a unit. The argument is based on the fact that acetylcholine produces similar percentage increases in the turnover of both phosphate and inositol, but, of course, the initial rates could be quite different. It does not seem possible to calculate these rates from the data presented by Hokin and Hokin.

Two other observations are relevant. The enzyme hydrolyzing phosphatidylinositol (195, 196), which is

widely distributed, removes inositol phosphate as a unit. On the other hand, in the currently accepted biosynthetic pathway for phosphatidylinositol, the phosphate comes from phosphatidic acid, while the inositol is incorporated independently.

The stimulation of phosphate turnover in phosphoinositides has been shown only in preparations such as tissue slices, where the cell membranes are intact. This bears out the suggestion of the Hokins that these lipids are connected with transport of substances out of the cell. In subcellular particles from guinea pig brain, acetylcholine increased only the incorporation of P^{32} into phosphatidic acid (215).

A high phosphate turnover has also been observed in the "phosphatido-peptide" fraction from various animal tissues (119). This fraction often contains phosphatidylinositol. Huggins (216) found that acetylcholine increased still more the incorporation of P^{32} into the phosphatido-peptide of chicken pancreas slices. Turnover of the total phospholipid fraction was increased about threefold, while that of the phosphatido-peptide increased sixfold. The acetylcholine concen-

TABLE 4. STIMULATION OF P^{32} TURNOVER IN PHOSPHOINOSITIDE AND PHOSPHATIDIC ACID OF TISSUE SLICES

Tissue	Stimulating Agent	Effect	P^{32} Ratio: Stimulated/Control		Reference
			Phospho-inositide	Phosphatidic acid	
Rabbit submaxillary gland	Acetylcholine 10^{-5}M	Secretion of mucin	5 (whole phospholipid fraction)		Hokin & Hokin (207)
Rabbit parotid gland	Acetylcholine 10^{-5}M	Secretion of amylase	4 (whole phospholipids)		Hokin & Sherwin (208)
Pigeon pancreas	Acetylcholine 10^{-3}M	Secretion of amylase	17	1.6; 3	Hokin & Hokin (181)
	Acetylcholine 10^{-4}M	Secretion of amylase	(a)* 9-18; (b) 2-18	2	Hokin & Hokin (97)
Pigeon pancreas	Pancreozymin 100 μg./ml.	Secretion of amylase	5 (whole phospholipids)		Hokin & Hokin (209)
	Secretin (0.005-50 units/ml.)	Secretion of H_2O, HCO_3-	No effect	No effect	
Sheep thyroid	Thyrotropic hormone (0.1 units/ml.)	Secretion of thyrotoxin ?	5.3	—	Freinkel (210)
Guinea pig brain	Acetylcholine 10^{-2}M	?	2.3	2.1	Hokin & Hokin (181, 81)
Guinea pig adrenal medulla	Acetylcholine 10^{-5}M	Secretion of epinephrine	2.5	2.0	Hokin *et al.* (211)
Rat adeno-hypophysis	CRF† 0.3 or 0.9 μg./ml.	Secretion of ACTH‡	1.4	1.4	Hokin *et al.* (212)
Rat adrenal	ACTH‡ 3.3 milliunits/ml.	Cortico-steroid synthesis and secretion	No effect	No effect	
Albatross salt gland	Acetylcholine 10^{-4}M	Secretion of NaCl	3.5	16	Hokin & Hokin (213)

* (a) and (b) are the two phosphoinositides separable on paper chromatograms.
† CRF, posterior pituitary corticotropin-releasing factor.
‡ ACTH, adrenocorticotropin.

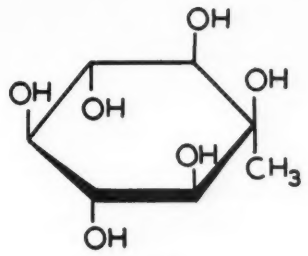
tration was 10^{-4}M. Acid-soluble phosphate turnover was unaffected. Huggins *et al.* (217) studied the P^{32}-labeled lipids of the phosphatido-peptide in greater detail. Chromatography on silica-impregnated paper or silicic acid columns revealed three components. One resembled phosphatidic acid and the other two were similar to phosphoinositides "a" and "b" of Hokin and Hokin (97).

It is possible that phosphatido-peptides are artifacts (123), due to nonspecific binding of acidic phospholipids by protein or peptide during the extraction procedures. These procedures are usually quite drastic, high speed homogenization of the tissue with 10 per cent trichloroacetic acid often being the initial step. It would be interesting to see whether the turnover of phosphoinositide in the phosphatido-peptide fraction is significantly different from the total phosphoinositide turnover in a given tissue.

Vladimirov *et al.* (218) equilibrated the acidified chloroform-methanol extract of P^{32}-labeled brain phosphatido-peptide with water. The phosphate compounds going into the aqueous phase had the highest specific activities, yet the inositol compounds remained in the chloroform. The exact nature of the various labeled phosphates has not been elucidated.

Moscatelli and Larner (219) injected C^{14}-inositol into rats and showed that after several hours the radioactivity appeared in exhaled CO_2, in liver glycogen, and in the tissue lipids. In starved rats, inositol incorporation into lipids was reduced by 90 per cent. Glucose and C^{14}-inositol were injected into a rat fed on a high protein and high carbohydrate diet. After 10 hours the animal was killed and the total lipids of brain, liver, kidney, and heart were isolated and counted. It was not proved that inositol was the only labeled lipid constituent, but it is at least likely to have been the major one. Moscatelli and Larner quote figures based on disintegrations per minute per mg. lipid. From data in the literature on phospholipid and inositol lipid contents of these tissues, the approximate relative specific activities of the tissue phosphoinositides can be calculated. Assuming that phosphatidylinositol is the only inositol lipid present, the relative specific activities would be: brain 17, liver 12, kidney 14, heart 85. The high turnover of heart phosphoinositides is noteworthy.

Posternak and Schopfer (220) studied the phospholipids of an inositol-requiring strain of *N. crassa*. Acid hydrolysis of the lipids released two different inositol phosphates separable by paper chromatography. Isomytilitol (XVI), which inhibited growth of the *N. crassa*, was found to be incorporated into the phospholipid in place of inositol. This interesting result may lead to an understanding of the mechanism of growth inhibition by isomytilitol.



(XVI)

OTHER ASPECTS OF METABOLISM AND FUNCTION

Under certain conditions (e.g., in fatty livers caused by biotin feeding) *myo*-inositol can act as a lipotropic agent. There is no definite evidence, however, that this lipotropic action is connected with the formation or metabolism of inositol phospholipids. It does not seem possible to produce fatty livers in rats by feeding diets deficient in inositol. With choline, which is a much more powerful lipotropic agent, this can easily be done. Deuel (221) has reviewed this subject and concludes that the mechanisms of action of choline and inositol as lipotropic agents are quite distinct.

McKibbin and Brewer (222) found that inositol feeding affected human plasma lipids very little. There were minor increases in total phospholipid, total cholesterol, and free cholesterol. Neutral fat apparently decreased.

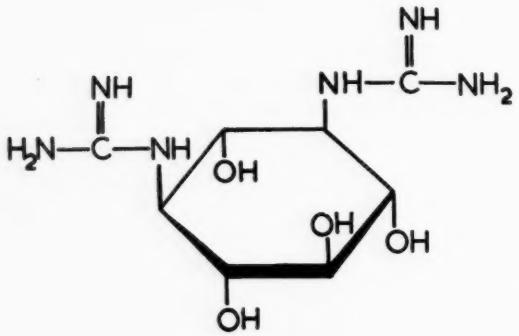
Needless to say, the effect of oral phosphoinositides on experimental atherosclerosis has been studied (223). The rats used had been fed on diets rich in cholesterol. The phosphoinositide had no effect. Inositol itself was similarly inactive (224).

Challinor and Daniels (225) studied the growth of *Saccharomyces cerevisiae* in media containing reduced levels of growth factors. When the inositol content of the medium was reduced from 10 μg. per ml. to 0.6 μg. per ml., the appearance of the yeast changed considerably and it produced much more fat (24.5 per cent as compared with 1.6 per cent based on dry weight). The nature of the fat produced and the mechanism of this dramatic effect are still unknown.

It was once thought that pancreatic amylase contained inositol which was essential for enzymic activity. Use of purer preparations has shown this to be untrue (references in 226), but Sarma and his col-

leagues (226, 227) have found an interesting relationship between inositol metabolism and amylase synthesis. In pigeon pancreas slices (227) ω-methyl pantothenic acid inhibited amylase activity, turnover of phospholipids, and synthesis of phosphoinositide. A relation between amylase synthesis and formation of inositol lipids was suggested. In several other biological systems inositol stimulated amylase synthesis (226). In the germinating cereal *ragi*, the pantothenic acid antagonist mentioned above reduced the level of amylase and of lipid inositol. Addition of pantothenic acid restored both to their original values.

Streptomycin is a derivative of 1,3-diguanido-*scyllo*-inositol (XVII). Its usefulness against *M. tuberculosis*



(XVII)

and the fact that this organism is rich in phosphoinositides have prompted the suggestion that the antibiotic may act by interfering with the metabolism of these lipids. Rhymer *et al.* (228) obtained some evidence that lipositol and a brain lipid fraction could suppress the activity of streptomycin against *S. aureus*.[10] Ata *et al.* (230) showed that *myo*-inositol was inactive against dihydrostreptomycin when the growth of *M. tuberculosis avium* was studied. However, after eight transfers in a medium containing 0.5-1 per cent *myo*-inositol, a streptomycin-resistant strain was obtained.

The remainder of this review must be largely speculative since so little is known of the function of phosphoinositides. Eagle *et al.* (39), who showed that *myo*-inositol is an essential growth factor for many human cells in tissue culture, suggest that its primary function may be in phosphoinositide metabolism. The concentrations of inositol required for optimum growth were higher than those expected of a typical vitamin or cofactor and point to a metabolic need for inositol.

The work of the Hokins, which has been summarized before, provides the soundest evidence so far with regard to function. It seems likely that the inositol lipids are concerned with the transport of materials across the cell membrane. Like all phospholipids, they may form part of many membranes within the cell, too, and it is unlikely that this role is merely structural (if indeed anything in a cell can be called merely structural).

After years of neglect, the phospholipids are beginning to be slightly fashionable in biochemical circles. One of the most promising fields in which they appear is that of the subcellular particles such as the electron transfer systems (128). The so-called "microsomal" fraction is also rich in phospholipid, and there are already (so far, doubtful) claims that phospholipids play a role in amino-acid activation for protein synthesis.

Murray *et al.* (231) have shown that the metaphase arrest caused in rat fibroblast culture by the mitotic poison colchicine could be reversed by *myo*-inositol. It is tempting to deduce from this that inositol derivatives play a part in the cell division process. However, tropolone, which has a seven-carbon ring structure, foreign (as far as we know) to animal tissues, is an even more powerful inhibitor of the colchicine effect (232). The possible role of inositol or its derivatives must therefore remain an open question. Soybean phosphoinositide (232) and liver phosphatidylinositol[11] were not effective in this system, but this may partly be due to the difficulty of getting such molecules inside cells.

Levin *et al.* (233) have studied phospholipid metabolism in rat liver following partial hepatectomy. As measured by P^{32}, the turnover of phospholipid increased with mitotic activity. This was due to a higher turnover of lecithin in all parts of the cell and a higher turnover of nuclear cephalin. Since cephalin from liver nuclei contains phosphoinositide, the latter may well take part in this stimulated activity.

From a chemical point of view it is paradoxical that phospholipids containing an unreactive cyclitol should be metabolically active. It is the inositol phosphate moiety in the phosphoinositides that holds most interest for the future. There is no doubt that this part of the molecule can be removed enzymatically, but we know little about the reasons for its removal or its further reactions with other cellular components. Chargaff (234) has speculated that it may act as a phosphate

[10] Later work failed to confirm this. (H. E. Carter, personal communication; also Reference 229.)

[11] H. H. Benitez, E. Chargaff, and J. N. Hawthorne, unpublished observations.

donor, combining, for instance, with nucleosides to give nucleotides and free inositol. Reactions of this sort would depend on the ability of hydroxy-compounds to form cyclic phosphate intermediates, as has been indicated already. Phosphate cyclization could be of great importance in the metabolism of inositol phospholipids.

Though an attempt has been made to make this review comprehensive, I must apologize for overlooking significant papers, particularly in languages other than English. My thanks are due to Professor Erwin Chargaff, in whose stimulating company I first met the phosphoinositides, and to Dr. Georg Hübscher for helpful discussion of the metabolic aspects. I am also grateful to Mrs. R. Williams for all her work in preparing the manuscript.

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Quantitative glass paper chromatography: a microdetermination of plasma cholesterol*

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SUMMARY

A rapid, sensitive method for the determination of sterols is described. Microgram samples are spotted on impregnated glass fiber paper, chromatographed for 7 minutes, dried, sprayed with sulfuric acid, and charred by heating. The amount of char formed is measured by densitometry. Values obtained for cholesterol in the serum are in agreement with those obtained by the Sobel-Mayer method. The method has been adapted to the determination of cholesterol in 10 μl. of plasma, and to the determination of standard solutions of other sterols.

Glass fiber paper has been used for qualitative chromatography because of its ability to withstand greater chemical and thermal stress than cellulose fiber paper. With glass fiber paper, it is possible to locate compounds on the chromatograms by spraying with sulfuric acid followed by heating to char the compounds. This technique has been applied to the separation of glycerides (1), phospholipids (2, 3, 4), sugars (5), methyl esters of the polybromosterates (6), saponins (7), toxic substances from tung oil (8), bile acids (9), steroids (10, 11), cholesterol and its esters (1), and triterpenoids (12). The chromatograms showed a gross relationship between quantity of the compound added and the amount of char formed.

MATERIAL AND METHODS

Preparation of Coated Glass Paper. Glass filter paper[1] is cut to 15 x 19 cm. size and a 0.4 cm. hole punched in the center of one 15 cm. side near the edge. Several papers, suspended by a glass rod through the holes, are heated in a furnace at 600°C for 30 minutes to clean the paper of organic matter. After this cleaning, and through all subsequent stages, care must be taken to avoid contamination with organic matter, such as fingerprints and dust.

Sodium silicate is applied to the glass paper in aqueous solution. A 2 per cent stock solution of sodium silicate is prepared with 21 ml. of sodium silicate solution (40°-42° Bé, Mallinckrodt), diluted to 400 ml. with distilled water, and from this a 0.4 per cent solution is made by dilution. The cleaned glass paper is dipped in the 0.4 per cent sodium silicate solution, a clean glass rod is passed across both surfaces to drain excess fluid, and a small binder clip is used to suspend the paper vertically over a hot plate to dry. The silicic acid paper is prepared according to the method of Dieckert *et al.* (13). The coated papers are stored in a clean covered container until used.

Preparation of Samples for Chromatography. The compounds used as reference standards were dissolved in suitable solvents and made up to contain 20, 40, 60, 80, 100, 120, 140, and 160 μg. per ml.

The extracts of serum used for comparison of this method with the Sobel-Mayer method (14) for total cholesterol were prepared by pipetting 1 ml. of serum into a 25 ml. volumetric flask, to which was added approximately 6 ml. of alcohol-ether solution 3:1 (v/v) as a fine spray from a hypodermic syringe and

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[1] No. X-934-AH, from Reeve Angel and Co., Clifton, N. J.

needle. The volumetric flask was heated to boiling in a water bath and cooled. Twelve drops of 33 per cent potassium hydroxide were added and thoroughly mixed, and the flask was incubated for 30 minutes at 37°C. After filling to the mark with the alcohol-ether solution, mixing, and allowing the protein to settle, a portion was decanted and saved in a stoppered vial. Ten μl. of this solution was applied to the chromatogram for the determination of cholesterol.

For the microdetermination of cholesterol in plasma, blood was obtained by clipping the tails of rats, collected in a heparinized capillary tube, one end sealed, and the tube was centrifuged to separate red cells from plasma. The capillary tube was broken so that 10 μl. of cell-free plasma could be collected in a micropipette. This was transferred to a small screw-cap vial, to which was added 1 ml. of alcohol-ether 3:1 (v/v), containing 3 gm. KOH per 100 ml. After mixing, it was incubated at 37°C for 30 minutes; the mixture was applied to the chromatogram in 10 μl. aliquots.

Chromatographic Procedure. As a guide to placement of samples, light dots were made with a lead pencil approximately 2 cm. apart and 1.5 cm. from the bottom edge of the paper. Samples and standards were applied at these points from a 10 μl. micropipette, using the same pipette for all samples. On each chromatogram the unknowns were spotted in duplicate with at least three different concentrations of standard solution. The pipette was cleaned with chloroform and dried with air after pipetting each sample. After the samples were spotted on the paper, they were allowed to dry at room temperature. The paper was suspended in a covered tank with the lower 3 mm. in the solvent. Approximately 7 minutes was sufficient for capillary action to bring the solvent front to 1 cm. from the top edge of the paper. The chromatogram was removed and the solvent evaporated by holding the paper horizontally over a hot plate until solvent odor could no longer be detected. The dried chromatogram was sprayed with fresh reagent grade concentrated sulfuric acid from an atomizer to coat both sides evenly but not heavily, and was hung vertically in an oven at 230°C for 4 minutes. This charred the compounds, producing a grayish spot approximately 14 mm. or less in diameter.

Description and Operation of the Densitometer. A special densitometer (Fig. 1) that measures the light absorbance of the entire 14 mm. spot was constructed. The unit consists of a variable intensity light source, a ground-glass diffusor, an unobstructed cylindrical light path, a holder for the paper with a light opening slightly larger than the spots, a shutter, and a 5581 phototube. The light filament, a 15-candlepower automotive type 1004, is 12 cm. from the sample, and the sample is 3 cm. from the center of the horizontally mounted phototube. The limiting light opening in the sample holder is 14 mm. in diameter. The phototube base is connected in parallel with shielded wires to the phototube base of a photometer; equally good results are obtained with the Bausch and Lomb Spectronic 20 or the Beckman Model C Colorimeter. In both cases the phototube is removed from the photometer and inserted into the densitometer attachment.

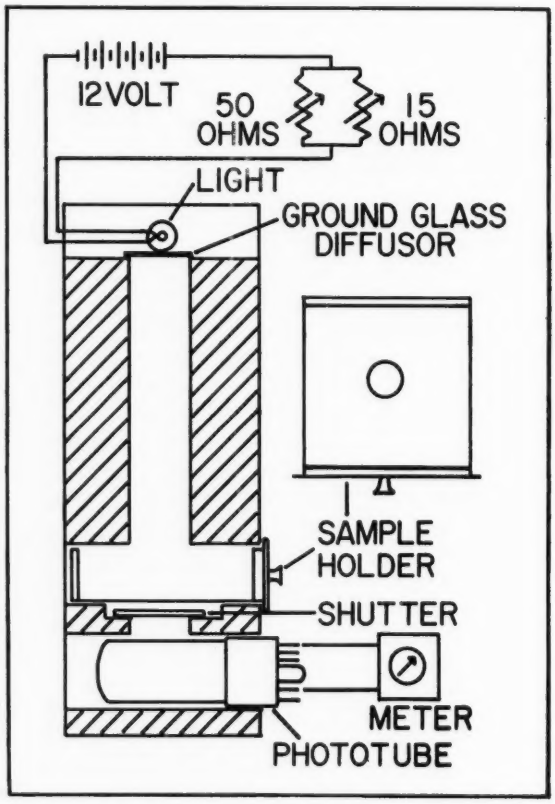

FIG. 1. Schematic drawing of the densitometer.

The meter is set to infinity by varying the dark current while the shutter is closed. Using a Teflon® blank, the densitometer is set to zero absorbance by varying the rheostats in the battery-light system. The blank consists of two thicknesses of Teflon®, 1/8 inch and one 1/16 inch, fastened together with small screws. The chromatogram is cut into pieces approximately 2 x 3 cm., each with one charred spot on it. A circle is

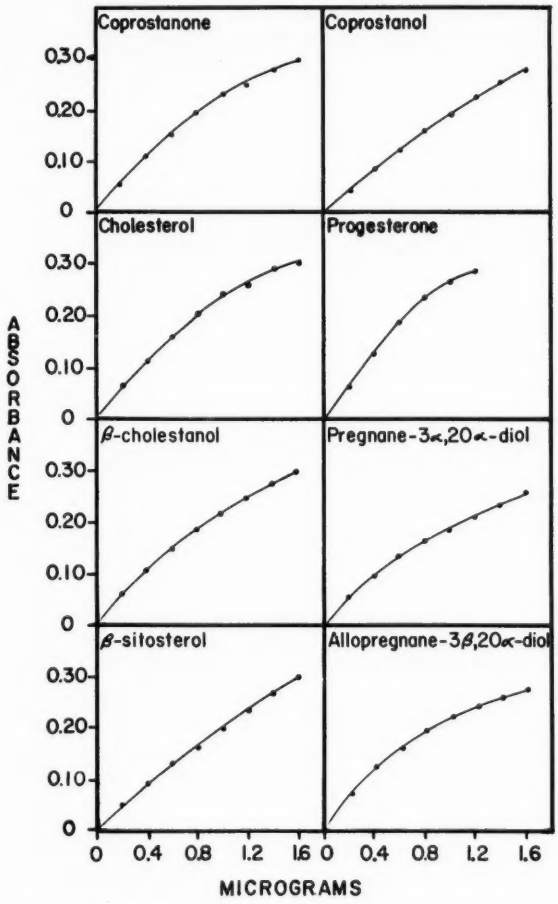

FIG. 2. Results of analyses of sterol reference standards by quantitative glass paper chromatography.

drawn with a soft lead pencil around the charred spot, 2 mm. larger in diameter than the light opening of the densitometer sample holder, in order to center the spot in the holder. At the same level on the chromatogram an additional piece of paper, without a charred spot, is cut for use as a blank. Each piece is placed on the holder so that the spot is centered over the light opening, a large metal or plastic washer is placed over the paper to press it flat on the sample holder, and the absorbance is measured. After this has been done for each of the spots, the small pieces of paper are placed on a hanger in the oven (600°C). The charred spots completely disappear in 15 minutes, but the pencil mark remains so that the paper may be centered again on the sample holder. Then the absorbance of these small pieces is determined for the second time, giving a value for the absorbance of the glass paper alone. This blank is necessary since the glass paper varies considerably in absorbance from area to area. Centering the spot on the sample holder is facilitated by working over a horizontally placed X-ray view box.

Calculation. The absorbance of a given sample is calculated by the formula below:

$A_s = (A_1 - A_2) - (A_3 - A_4)$

A_s = Absorbance of sample

A_1 = Absorbance of glass paper with charred spot

A_2 = Absorbance of glass paper after removing charred spot

A_3 = Absorbance of glass paper blank

A_4 = Absorbance of glass paper blank after removing the char of the reagents

The absorbance of the standards is plotted on linear graph paper. The amount of material in the test solution is read from this graph.

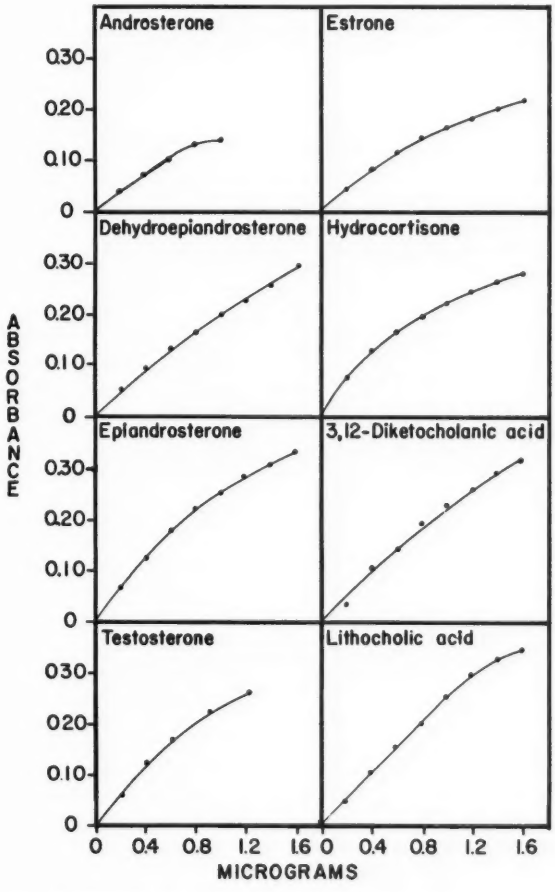

FIG. 3. Results of analyses of sterol reference standards by quantitative glass paper chromatography.

RESULTS

Figures 2 and 3 demonstrate standard curves obtained with a variety of sterols from solutions containing 20 to 160 μg. per ml. These standard curves were reproducible; nevertheless, standards and unknowns were always run together. Table 1 gives the adsorbants and solvents used to obtain the standard curves.

Two samples of pooled serum, one high in cholesterol concentration and one of normal value, were analyzed by this method and by the method of Sobel and Mayer. The normal serum, analyzed by the present method, had an average value of 187 mg. per 100 ml. (s.d. 9.3 mg., 50 determinations). The value obtained by the Sobel-Mayer method was 179 mg. per 100 ml. The serum high in cholesterol concentration had an average value by this method of 496 mg. per 100 ml. (s.d. 21.1 mg., 50 determinations). The value obtained by the Sobel-Mayer method was 474 mg. per 100 ml. Ten random samples of serum were analyzed by both methods and the results were in good agreement (Table 2).

TABLE 1. SOLVENT SYSTEMS AND R_f VALUES FOR COMPOUNDS IN FIGURES 2 AND 3*

Compound	Solvent System (v/v)	R_f Value
Coprostanone	Isooctane	0.88
Cholesterol	Isooctane	0.54
β-cholestanol	Isooctane	0.54
β-sitosterol	Isooctane	0.54
Coprostanol	Isooctane	0.70
Progesterone	Benzene, isooctane (1/1.5)	0.71
Pregnane-3α, 20α-diol	Benzene	0.29
Allopregnane-3β-20α-diol	Benzene, isooctane (1/1)	0.57
Androsterone	Benzene, isooctane (2/1.25)	0.47
Dehydroepiandrosterone	Benzene, isooctane (1/1)	0.59
Epiandrosterone	Benzene, isooctane (1/1)	0.66
Testosterone	Benzene, isooctane (1/1)	0.38
Estrone	Benzene, isooctane (1/1)	0.54
Hydrocortisone	Benzene, ethanol (100/1)	0.50
3,12-diketocholanic acid	Isooctane, acetic acid (200/7)	0.64
Lithocholic acid	Isooctane, acetic acid (200/3)	0.50

* The adsorbant for 3,12-diketocholanic acid was silicic acid and for the remainder was sodium silicate.

DISCUSSION

This method of quantitative glass paper chromatography is dependent upon certain features of the instrument and of the glass paper. The light used in the system must be variable in intensity and constant in beam width. The largest area of paper that can be analyzed without a focusing lens system is determined by the size of the light sensitive plate in the phototube. The light opening of the sample holder should be centered and the charred spot must lie directly in the effective light path. This effective light path, by encompassing the entire area of the charred spot, eliminates the need for motion inherent in slit-beam densitometry. The electronic part of the densitometer requires a suitable voltage supply to the phototube, a system of amplification, and a meter. The Beckman Model C Colorimeter and Bausch and Lomb Spectronic 20 work equally well when connected to a 5581 phototube in the densitometer. It is to be expected that other types of photometric apparatus can be adapted to this type of measurement. A reference blank is necessary due to the nonuniformity of the glass paper. Any material with constant light-absorbing qualities is suitable when its absorbance is less than that of the papers being analyzed. Teflon® proved ideal for this purpose.

The optimum conditions for developing the char depend upon temperature, length of heating time, and the chemical properties of the compound. Uniform conditions must be maintained in order that all the spots on the same paper are charred to the same extent. Due to the difficulty of maintaining a precise temperature while loading the oven, standard samples are run on each chromatogram to minimize errors. The air or other gas used to spray the sulfuric acid must be free of organic compounds. Many different sprayers and various spraying techniques give good results.

The method described has certain advantages. It is rapid and reproducible. The cholesterol determinations showed reasonable accuracy. A single determination can be completed in 45 minutes from the time of application of the sample to the chromatogram. Thus a technician can analyze at least 30 samples a day in duplicate. Because small samples are used for each determination (~ 1 μg.), this method can be adapted to microdeterminations of compounds of biological interest for which currently available methods are inadequate. The chromatographic separation reduces preliminary purification steps and is helpful in identification. Determination of compounds on chromatograms by charring is often more sensitive than methods

TABLE 2. COMPARISON OF TOTAL CHOLESTEROL CONCENTRATIONS OF TEN SERUM SAMPLES ANALYZED BY GLASS PAPER CHROMATOGRAPHY AND BY SPECTROPHOTOMETRY

Glass Paper Chromatography	Sobel-Mayer Method
mg./100 ml.	*mg./100 ml.*
220	240
259	258
215	196
221	229
178	175
204	185
185	193
200	190
142	135
198	183

depending on color development. The nonspecificity of the charring reaction makes it applicable to most carbon-containing compounds, but homogeneity of the spots to be analyzed must be established by accepted techniques.

In addition to the applications presented above, this technique has been applied to the determination of phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylethanolamine in serum (15, 16), as well as bile acids in feces (17). The procedure is being developed for the determination of estrogens in urine.

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Studies on the biosynthesis of cholesterol: XII. synthesis of allyl pyrophosphates from mevalonate and their conversion into squalene with liver enzymes*

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SUMMARY

The biosynthesis of allyl pyrophosphates (the pyrophosphates of dimethylallyl alcohol, geraniol, and of farnesol) from DL-mevalonate-2-C^{14} and from (-)5-phosphomevalonate-2-C^{14} with soluble liver enzymes in the presence of ATP and Mg^{++} is described. The allyl pyrophosphates were partially purified and their properties studied. They are unstable below pH 5 and cleave into inorganic pyrophosphate (identified by the use of purified yeast inorganic pyrophosphatase) and allylic alcohols. The alcohol components were identified by gas-liquid radiochromatography after hydrolysis of the pyrophosphates by prostatic, intestinal, microsomal, and snake venom phosphatases. Farnesyl pyrophosphate, which was the principal product among the allyl pyrophosphates, was shown to be the precursor of squalene in the liver enzyme system just as was reported for yeast. The enzyme or enzymes responsible for the conversion of farnesyl pyrophosphate into squalene are attached to microsomal particles and need either TPNH or DPNH and a divalent cation (Mg^{++}, Mn^{++}, or Co^{++}) as cofactors. Slightly better yields of squalene were obtained with TPNH than with DPNH. The squalene synthesizing system was strongly inhibited by p-chloromercuribenzoate and N-ethyl maleimide, but was not affected by iodoacetamide; the optimal pH was 7.4. Addition of soluble enzymes to this system reduced the yield of squalene and caused some conversion of the allyl pyrophosphates into carboxylic acids.

Recent studies on the enzymic synthesis of squalene from mevalonic acid (3,5-dihydroxy-3-methylpentanoic acid) in yeast extracts have revealed a number of new intermediates. 5-Phosphomevalonate (1), 5-diphosphomevalonate and 3-methylbut-3-en-1-yl pyrophosphate (isopentenyl pyrophosphate) have been recognized as successive intermediates in the synthesis of squalene (2 to 5). Furthermore, Lynen *et al.* (4) demonstrated that a protein fraction of yeast extracts catalyzed the synthesis from mevalonate of farnesyl pyrophosphate which was converted into squalene on addition of yeast cell particles and reduced pyridine nucleotides. More recently it was shown that 3,3-dimethylallyl pyrophosphate and geranyl pyrophosphate are intermediates preceding the formation of farnesyl pyrophosphate (6).

Experiments with liver enzymes have indicated that the reactions of squalene biosynthesis in mammalian cells are similar to those occurring in yeast cells. 5-Phosphomevalonate and 5-diphosphomevalonate and the enzymes responsible for their formation in liver extracts have already been described (7 to 10). In the present paper we report the synthesis, from mevalonate-2-C^{14} and from (-)5-phosphomevalonate-2-C^{14}, of acid-labile hydrophilic derivatives of dimethylallyl alcohol, geraniol, and of farnesol with soluble liver enzymes. These substances have been identified as pyrophosphate esters and some of their properties defined. Detailed studies on the synthesis of squalene from farnesyl pyrophosphate are also described. Brief accounts of these experiments have already appeared (11, 12).

* The following abbreviations are used: DPN and DPNH, diphosphopyridine nucleotide and its reduced form; TPN and TPNH, triphosphopyridine nucleotide and its reduced form; G-6-P, glucose 6-phosphate; ATP, adenosine triphosphate; EDTA, ethylene diamine tetraacetate; BAL, 2,3-dimercaptopropanol; GSH, glutathione; PCMB, p-chloromercuribenzoate.

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METHODS AND MATERIALS

Enzyme Preparations. Rat liver homogenates were made, as described in detail previously (13), in 0.1 M potassium phosphate buffer, pH 7.4 containing 5 mM $MgCl_2$ and 30 mM nicotinamide following the method of Bucher and McGarrahan (14). The preparation of microsomal and soluble enzyme fractions from the homogenates has also been described (15). After sedimentation of the microsomes at 105,000 $\times$ *g*, these cell particles were washed by suspension in buffer from which nicotinamide had been omitted (0.1 M potassium hydrogen phosphate buffer, pH 7.4, with 5 mM $MgCl_2$). After resedimentation at 105,000 $\times$ *g* for 45 minutes, the washed microsomes were suspended in a volume of the same buffer equal to one-tenth the volume of the liver homogenate from which they had been prepared. Nicotinamide was omitted during washing of microsomes in order to ensure destruction of pyridine nucleotides remaining attached to the particles.

The soluble supernatant of the rat liver homogenate was fractionated by the addition of solid ammonium sulfate. The active enzyme preparation used was that protein fraction which precipitated between 30 and 60 per cent ammonium sulfate saturation (F_{30}^{60}-enzymes). The precipitate was collected by centrifuging at 10,000 $\times$ *g* for 20 minutes; it was dissolved in 0.02 M $KHCO_3$ and dialyzed against the same for 3 to 4 hours. After clarifying the dialyzed solution by centrifugation, its protein content was 30 to 50 mg. per ml.

Incubations. These were made at 37°C, usually for 1 or 2 hours. One ml. anaerobic incubations were made in small ground-glass stoppered tubes, which were flushed with N_2 before and after filling and then securely stoppered. Larger anaerobic incubations were made under a continuous stream of N_2.

Extraction and Separation of Incubation Products. Assay of Squalene. The incubations were usually terminated by the addition of an equal volume of 15 per cent KOH (w/v) in 85 per cent ethanol. When the synthesis of squalene was measured, 10 mg. of carrier squalene was also added, and the mixture saponified by heating at 70°C for 1 hour. The unsaponifiable compounds were extracted with petroleum ether (b.p. 40°-60°C) and the squalene separated by chromatography on alumina washed with methylformate (16), squalene being eluted with petroleum ether (17). In one experiment squalene was further identified through the thiourea adduct. To the chromatographed specimen (after evaporation of the solvent) a few milliliters of a saturated methanolic solution of thiourea and a few milligrams of finely powdered thiourea were added. After thorough shaking the mixture was left at room temperature for a few days. The crystals of the clathrate were decomposed with water and the squalene extracted with petroleum ether.

Assay of Allyl Pyrophosphates and Carboxylic Acids. The simplest assay of the allyl pyrophosphates synthesized from C^{14}-labeled precursors was based on their stability toward alkaline hydrolysis and their instability in the presence of acid (see Results) as they cleave completely at pH 1 to 2 into inorganic pyrophosphate and alcohols, the latter being readily extractable with petroleum ether. After extraction of unsaponifiable substances from the saponified incubations (see preceding paragraph), 2 mg. of a mixture of dimethylallyl alcohol, geraniol, nerolidol, and of farnesol was added and the pH of the digest adjusted to 1 to 2 with HCl (or H_2SO_4). After one to several hours the mixture was made alkaline (pH 10 or above) with aqueous KOH and the free alcohols extracted with petroleum ether. Carboxylic acids were finally extracted by acidifying again the aqueous phase and extracting the acids with petroleum ether.

Assay of C^{14}. This was done with a thin mica-window Geiger-Müller counter (window thickness 1.2 mg. per cm.^{2}), with an efficiency of about 6 per cent. A given petroleum ether extract was first evaporated to a measured volume and an aliquot of this (usually one-tenth) plated on a plastic planchette of 2 cm.^{2} area lined with lens paper. All counts were made at negligible thickness (less than 1 mg. per cm.^{2}). With samples of free alcohols the planchettes were counted immediately upon evaporation of the petroleum ether at room temperature, so as to minimize loss of counts because of the volatility of these alcohols. In the presence of carrier alcohols the counting rate of a given planchette diminished by less than 5 per cent in the first 20 minutes; experiments with known solutions have demonstrated that quantitative analyses can be achieved by this technique.

Analysis of Alcohols and Acids. Gas-liquid radiochromatography (18) was the method employed for the identification of both alcohols and acids produced from mevalonate-2-C^{14}. Acids were methylated with diazomethane before analysis. Both polar (ethylene glycol-adipate-polyester) (19) and nonpolar (Apiezon-L vacuum grease) stationary phases were used in the gas-liquid chromatographic columns with theoretical plate efficiencies of 2700 to 3600 measured with methyl stearate. Most of the analyses were done on polar stationary phase columns at 197°C. The identification of the primary and tertiary allylic alcohols

(prenols) from C_5 to C_{20}, and of the carboxylic acids (prenoic, or terpenoid acids) corresponding to the primary prenols, by the use of these two types of columns in gas-liquid chromatography has been described in detail (20). With the gas-liquid radiochromatographic technique one obtains a simultaneous recording of the analysis of a volatile mixture with a gas-density meter (21) together with that of the total C^{14}-content of any one component. The radiochromatographic record appears as discrete steps (integral shape record, Fig. 1) corresponding in location to the peaks recorded with the gas-density balance; the increment in counting rate in a given step indicates the total radioactivity associated with that particular fraction. The resolving power of the columns was such that not only could all the alcohols be completely separated but also their geometric isomers (e.g., *cis-trans-* and *trans-trans-*farnesol).

Phosphatase Preparations. Yeast inorganic pyrophosphatase was generously provided by Dr. P. Hele of this Unit; it had been purified by the method of Kunitz (22) to the stage immediately before crystallization, and stored at −20°C after precipitation with ammonium sulfate. For use the precipitate was dissolved in, and was dialyzed against, 0.02 M 2-amino-2-hydroxymethylpropane-1,3-diol-HCl (tris-HCl) buffer, pH 7.4. Incubations with this enzyme were made in 0.1 M tris-HCl buffer, pH 7.2 at 30°C for 15 minutes.

Purified calf-intestinal alkaline phosphatase was the product of C. F. Boehringer & Soehne, Mannheim, Germany. One hundred mg. of the freeze-dried preparation was dissolved in 5 ml. of 1 per cent bovine serum albumin solution and up to 0.5 ml. of this was used in incubations in 0.1 M tris-HCl buffer, pH 8.6.

Prostatic acid phosphatase was a 5-year-old preparation purified by the method of London and Hudson (23) from normal human prostates obtained from post-mortems. When prepared, the enzyme had a specific activity some three hundredfold greater than the best preparation of London and Hudson (23) and was free of proteolytic activity. The enzyme, precipitated with ammonium sulfate, had been stored at −20°C; it largely retained its activity. It was dissolved in and dialyzed before use against the same buffer as the yeast phosphatase. It was used in 0.1 M tris-acetate buffer, pH 5.7.

Snake venom *(Naja flava)* was obtained from the South African Institute for Medical Research, Johannesburg, and was used, after dissolving in 0.01 M $KHCO_3$, at pH 8.3 in 0.1 M tris-HCl buffer. It hydrolyzed the allyl pyrophosphates very slowly; at a concentration of 5 mg. per ml. only 10 per cent (0.15 μmole) of 1.5 μmoles of farnesyl pyrophosphate being hydrolyzed in 1.5 hours at 30°C. Even after 48 hours of action, only 85 per cent hydrolysis could be achieved with this preparation.

In all incubations with phosphatases Mg^{++} ions were present in a concentration of 5 mM. Hydrolysis of the allyl pyrophosphates with the prostatic and intestinal phosphatases was made in incubations at 37°C for 2 to 3 hours which were then continued at room temperature (22°C) for 16 to 20 hours to ensure complete hydrolysis. Free alcohols released during these incubations were extracted with petroleum ether after adjusting the pH of the incubations to about 10 and after the addition of 2 mg. of carrier alcohols.

Other Methods. Protein concentrations were determined with the biuret method (24). Inorganic orthophosphate was measured by the method described by London and Hudson (23); inorganic pyrophosphate is not detected by this method.

Materials. The sources of most of the purified coenzymes (TPN, DPN, ATP, and G-6-P) were given in a previous communication (25). Purified TPNH (enzymically reduced) and G-6-P dehydrogenase were the products of C. F. Boehringer and Soehne. Bovine plasma albumin, GSH, EDTA, BAL, and N-ethylmaleimide were obtained from L. Light and Co., Colnbrook, England.

The various inorganic salts and organic solvents and reagents were all analytical grade chemicals. Collidine (2,3-dimethyl pyridine) and petroleum ether were redistilled before use.

TABLE 1. SYNTHESIS OF ALLYL PYROPHOSPHATES FROM DL-MEVALONATE-2-C^{14} WITH SOLUBLE ENZYME FRACTION OF RAT LIVER*

Enzyme System	Allyl Pyrophosphates
	cpm.
Complete	30,220
No Mg^{++}	19
No ATP; + ADP (30 μmoles)	2,400
+ TPNH† (2 μmoles)	27,690
+ TPN (2 μmoles)	25,130
+ TPNH† + DPNH (2 μmoles each)	25,170

* Anaerobic incubation for 1 hr. at 37°C. The complete system contained: F^{60}_{30}-enzymes, 25 mg. protein/ml.; 7.5 mM ATP; 5.0 mM $MgCl_2$; 0.1 M potassium hydrogen phosphate buffer, pH 7.4; 0.01 M NaF; and 5 μmoles of DL-mevalonate-2-C^{14} (140,000 cpm. at negligible thickness), in a total volume of 4 ml.

† Generated with G-6-P and glucose-6-phosphate dehydrogenase present in the F^{60}_{30}-preparation.

DL-Mevalonic acid-2-C^{14} lactone was purchased from the Radiochemical Centre, Amersham, Bucks, England, and had been prepared by the method of Cornforth *et al.* (13). It was diluted with recrystallized, unlabeled mevalonic acid lactone before use, to give a solution with a specific activity of 0.2 mc. per mmole. As the lactone is inactive in enzyme reactions (10), the potassium salt of mevalonic acid was prepared by mild hydrolysis with KOH. Levorotatory 5-phosphomevalonate-2-C^{14} (26) was prepared by Levy and Popják (10) with purified liver mevalonic kinase; its specific activity was 32,000 cpm. per μmole (0.2 μc. per μmole) counted at negligible thickness.

Specimens of dimethylallyl alcohol, nerol (*cis*-geraniol), farnesol, nerolidol, and of squalene were provided by colleagues acknowledged at the end of this paper. Linalool was the product of A. Boake Roberts and Co., London, England, whose product "Meranol," which is pure geraniol, was also used. The sources and preparation of the various prenoic acids are described elsewhere (20).

RESULTS

Enzymic Synthesis of Allyl Pyrophosphates. When the F_{30}^{60}-enzymes were incubated with DL-mevalonate-2-C^{14}, ATP, and Mg^{++} ions aerobically or anaerobically, in the presence or absence of reduced pyridine nucleotides, only traces of radioactivity could be extracted with petroleum ether from the saponified incubation mixture. After acidification of the alkaline digests followed by neutralization, however, C^{14}-labeled neutral substances could be readily extracted. The results of a typical experiment, shown in Table 1, demonstrate that only ATP and Mg^{++} ions, and no pyridine nucleotides, are needed for the formation of these substances. With DL-mevalonate as substrate, the yield has varied from 20 to 40 per cent. With the optically active (-)5-phosphomevalonate as substrate, however, the yield was 75 to 80 per cent.

Gas-liquid radiochromatographic analyses of the neutral products extractable with petroleum ether, after acidification of the incubation mixtures, revealed a number of volatile substances different from any hitherto recognized animal product. Their origin from mevalonate suggested that they might be isoprenoid substances containing the characteristic grouping: $-CH_2-C(CH_3)=CH-CH_2$. Subsequently they were identified as C_5, C_{10}, and C_{15} allylic alcohols, since the radioactive fractions had, on columns with polar and nonpolar stationary phases, retention volumes indistinguishable from those of authentic marker substances added to the biosynthetic specimens (see further under Identification of the Allyl Alcohols).

It followed from the mode of isolation of the alcohols that they did not accumulate in the enzyme incubations in the free form, but as derivatives resistant to alkaline hydrolysis and very unstable in the presence of acid.

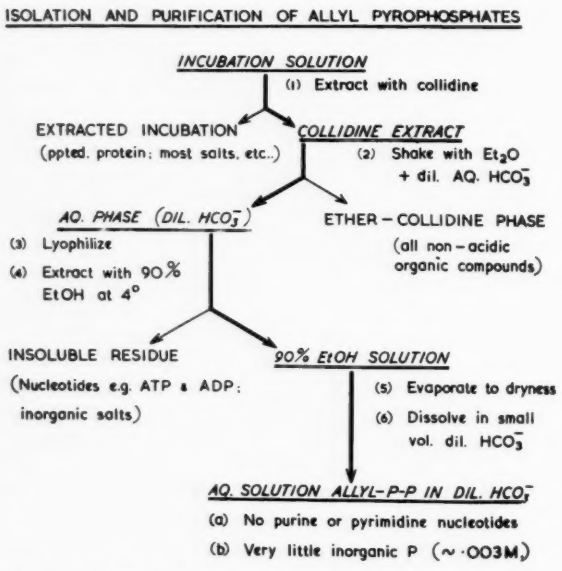
Further experiments showed that iodoacetamide in concentrations of 5 and 10 mM prevented completely the formation of these acid-labile derivatives of the allylic alcohols in incubations identical with the complete system shown in Table 1.

In all experiments on the synthesis of the acid-labile derivatives of the allylic alcohols with the F_{30}^{60}-enzymes, the incubations were made anaerobically and contained the same components, in the same concentrations, as listed in Table 1 for the complete enzyme system. When large-scale experiments were made for preparative purposes, multiples of this basic 4 ml. incubation mixture were set up and the incubations lasted for 2 hours, with additional amounts of ATP (5 μmoles per ml.) added at the end of the first hour.

Isolation and Partial Purification of Derivatives of Allylic Alcohols. The derivatives of the allylic alcohols could be extracted in an unhydrolyzed form from the enzymic incubations with collidine. A 28 ml. reaction mixture was set up with F_{30}^{60}-enzymes with 35 μmoles of DL-mevalonate-2-C^{14} as substrate. After 2 hours of incubation, a 4 ml. sample was assayed in the usual way (after saponification, acidification, etc.) for the amount of allylic derivatives formed; 40,980 cpm., equivalent to a conversion of 1.46 μmoles of mevalonate, were found in allylic alcohols in this 4 ml. sample. The remaining 24 ml. of the incubation was extracted three times with 20 ml. of collidine, the aqueous phase and the precipitated protein being separated by centrifugation. To the combined collidine extracts (75 ml.) 150 ml. of 0.02 N aqueous KOH and 100 ml. of ether were added in a separatory funnel and the mixture shaken. After separation of the phases, the ether-collidine layer was drawn off, and the extraction of collidine remaining in the aqueous phase completed by three further extractions with ether (100 ml. each). The combined ether extracts contained no radioactivity. The yellowish aqueous phase (86 ml.) contained all the radioactivity of the initial collidine extract. After removing the ether dissolved in the aqueous phase with a stream of nitrogen, the preparation was dried from the frozen state to yield a fine brownish powder. This powder was extracted in small portions with a total of 10 ml. of 80 per cent ethanol. This ethanolic solution, clarified by centrifuging, was

concentrated to 2 ml. under a stream of N_2 at room temperature (allyl pyrophosphate preparation No. 1). Extraction of this concentrate with petroleum ether removed only small amounts of radioactivity (equivalent to the C^{14}-content of about 0.001 μmole of mevalonate). One-fifth of the aqueous solution (after extraction with petroleum ether) was acidified with H_2SO_4 (pH about 1) and then after about 30 minutes was made alkaline and extracted again with petroleum ether. This extract now contained 48,827 cpm. Thus, through the whole process of collidine extraction, etc., $5 \times 48{,}827 = 2.441 \times 10^5$ cpm. of acid labile C^{14}-compounds were obtained from 24 ml. of incubation, corresponding to the conversion of 8.7 μmoles of mevalonate. From the direct analysis of the 4 ml. aliquot of the original 28 ml. incubation, the expected value was 2.459×10^5 cpm. Gas-liquid radiochromatographic analysis of the petroleum ether extracts of the acidified 4 ml. aliquot of the original incubation and of the final petroleum ether extract of the specimen prepared through collidine extraction revealed the same allylic alcohol components in identical ratios.

The method of isolation and partial purification of the derivatives of the allylic alcohols given in Scheme 1 is based on experience gained during the experiment


ISOLATION AND PURIFICATION OF ALLYL PYROPHOSPHATES
INCUBATION SOLUTION
(1) Extract with collidine
EXTRACTED INCUBATION
(ppted. protein; most salts, etc..)
COLLIDINE EXTRACT
(2) Shake with Et2O + dil. AQ. HCO3-
AQ. PHASE (DIL. HCO3-)
ETHER – COLLIDINE PHASE
(all non – acidic organic compounds)
(3) Lyophilize
(4) Extract with 90% EtOH at 4°
INSOLUBLE RESIDUE
(Nucleotides e.g. ATP & ADP; inorganic salts)
90% EtOH SOLUTION
(5) Evaporate to dryness
(6) Dissolve in small vol. dil. HCO3-
AQ. SOLUTION ALLYL-P-P IN DIL. HCO3-
(a) No purine or pyrimidine nucleotides
(b) Very little inorganic P (~ ·003M)

SCHEME 1. Flow sheet of the purification of allyl pyrophosphates.

just described, and was used during later experiments. The progress of the purification is indicated by the italics and heavy arrows. The compounds were first extracted from the enzymic incubations with collidine, and their aqueous solution prepared by shaking the collidine extract with diethyl ether and dilute aqueous bicarbonate (in preference to KOH). The solubility of the compounds in water at neutral or slightly alkaline pH is considerably greater than in collidine, so that several extractions with excess volume of collidine were necessary to achieve a quantitative extraction of the products from the incubation mixtures. They are practically insoluble in an ether-collidine mixture (2:1, v/v) so that on the addition of 2 volumes of ether and 0.5 volumes of 0.01 M aqueous $KHCO_3$ to the collidine extract, the compounds could be quantitatively transferred into the aqueous phase. Contaminating nucleotides and salts were then removed by precipitation of these at 4°C by the addition of ethanol to a final concentration of 90 per cent. After evaporation of the ethanol at room temperature under reduced pressure, the compounds were dissolved in 0.01 M aqueous $KHCO_3$ and stored at —20°C.

For further studies a large-scale, 2-hour incubation (75×4 ml.) was set up with 8 g. of F_{30}^{60}-enzymes and with a total of 300 μmoles of (-)5-phosphomevalonate-2-C^{14} as substrate. The derivatives of the allylic alcohols were isolated from this incubation according to the procedure outlined in Scheme 1; assay of an aliquot of the incubation mixture showed that 240 μmoles of the substrate added were converted into these substances. Unless stated otherwise, the experiments still to be described were made with this preparation (allyl pyrophosphate preparation No. 3).

Identification of the Acid-Labile Derivatives of Allylic Alcohols as Pyrophosphate Esters. Lynen *et al.* (4) noted that farnesyl pyrophosphate when exposed to 5 per cent cold trichloroacetic acid decomposed into inorganic pyrophosphate and an organic residue. The instability in the presence of acid of the derivatives of the allylic alcohols formed in liver enzyme incubations suggested that these also might be pyrophosphate esters. To test this possibility an aqueous solution of the compounds (preparation No. 3) was first acidified in order to liberate the free alcohols (see below). The solution was then neutralized, and the alcohols extracted with petroleum ether. The number of micromoles of alcohols released was calculated from the C^{14}-content of the petroleum ether extract, the gas-liquid radiochromatographic analysis of the alcohols, and from the known specific activity of the 5-phosphomevalonate-2-C^{14} from which they had been synthesized. The specific activity (counts per minute per μmole) of the C_{15}-alcohols (farnesol and nerolidol) was taken as three times, and

that of the C_{10}-alcohols as twice that of the 5-phosphomevalonate. One-half of the aqueous phase extracted with petroleum ether was analyzed directly for inorganic orthophosphate. The other half was incubated with yeast inorganic pyrophosphatase—which splits inorganic pyrophosphate into orthophosphate—and then also analyzed for orthophosphate. As is shown in Table 2, incubation with pyrophosphatase

TABLE 2. IDENTIFICATION OF ALLYL ALCOHOL DERIVATIVES AS PYROPHOSPHORIC ACID ESTERS*

Treatment	μmole Orthophosphate /μmole Alcohol
(*a*) Acidify and then neutralize	1.4
(*b*) Acidify and neutralize + pyrophosphatase treatment	3.0
(*c*) Pyrophosphatase alone†	1.8

Pyrophosphate released by acid = $0.5 \times (b - a)$ = 0.8 μmole/μmole alcohol

* Each analysis was made on 0.2 μmole of allyl alcohol derivatives (prep. No. 3). See also text.

† It is probable that the small increase in orthophosphate as compared to (*a*) is due to slight hydrolysis of allyl pyrophosphates by yeast phosphatase. These compounds have been hydrolyzed, although sometimes quite slowly, by every phosphatase tested so far.

released an additional 1.6 μmoles of orthophosphate (equivalent to 0.8 μmole of pyrophosphate) per μmole of alcohol released. The amount of orthophosphate listed in line *a*, 1.4 μmoles per μmole of alcohol, is considered to represent the inorganic phosphate content of the partially purified specimen. To prove that the effect of pyrophosphatase treatment was not due to the presence of preformed inorganic pyrophosphate in the solution, a third aliquot was incubated with the pyrophosphatase without previous acidification. As shown in line *c*, this resulted in only a very small increase in the content of orthophosphate. These data therefore show that acidification releases almost 1 mole of inorganic pyrophosphate per mole of alcohol, from which we conclude that the original compounds are alcohol pyrophosphates.

Identification of the Allyl Alcohols. The organic moieties of the pyrophosphate esters were identified as allylic alcohols by gas-liquid radiochromatographic analysis. Samples of the pyrophosphate esters (allyl pyrophosphate preparations No. 1 and No. 3) were hydrolyzed with various phosphatases for sufficient length of time to ensure complete hydrolysis (see Methods). The enzymic hydrolysis was judged complete when the amount of C^{14} extractable with petroleum ether after such hydrolysis was identical with the amount of C^{14} extractable after acid hydrolysis. Figure 1 illustrates typical gas-liquid radio-

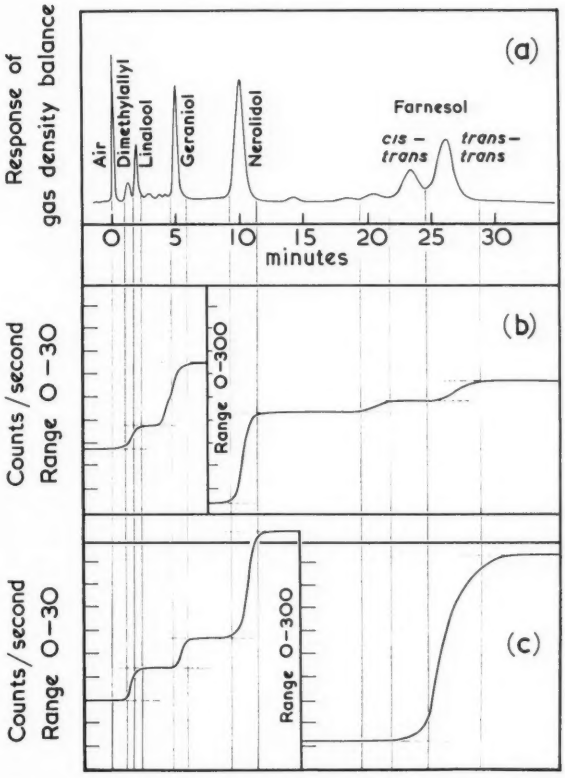


FIG. 1. Gas-liquid radiochromatographic analysis of the alcohols released from an aqueous solution of allyl pyrophosphates (see text). Ethylene glycol-adipate polyester stationary-phase column; 197°C. Record (*a*) shows the analysis of a reference mixture of unlabeled alcohols added as carrier; record (*b*) shows the simultaneous radiochromatographic analysis of the alcohols released by acid hydrolysis; and record (*c*) shows the alcohols released by enzymic hydrolysis, with prostatic acid phosphatase.

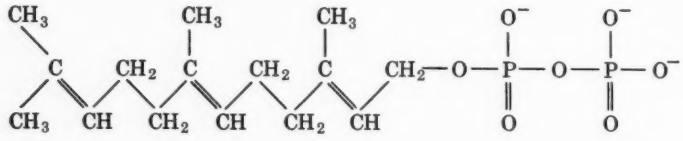
chromatographic analyses of allylic alcohols on ethylene glycol-adipate polyester column: (*a*) is the analytical record (gas-density meter response) of authentic marker substances added to the biosynthetic specimens; (*b*) and (*c*) are the radiochromatographic records of the organic components of allyl pyrophosphate preparation No. 3 hydrolyzed by acid and by prostatic acid phosphatase respectively. Records (*b*) and (*c*) were obtained of course in two separate runs (with identical markers), but for purposes of illustra-

tion were combined into one figure. In records (*b*) and (*c*) certain radioactive fractions correspond exactly with the analytical peaks shown in record (*a*). Since similar correspondence was found during analyses on another type of column (stationary phase Apiezon-L vacuum grease), the radioactive components may be assigned with confidence to some of the marker substances.

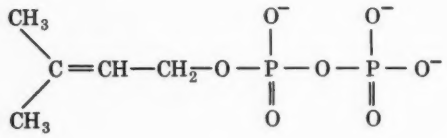
After hydrolysis with prostatic phosphatase, almost 90 per cent of the radioactivity was associated with *trans-trans* farnesol, about 6 per cent with the tertiary C_{15}-alcohol nerolidol, and smaller amounts with geraniol and 3,3-dimethylallyl alcohol (Fig. 1*c*). There were also present small radioactive fractions with retention volumes greater than that of farnesol. After hydrolysis with acid, however (Fig. 1*b*), a distinctly different pattern was obtained. Here, as after enzymic hydrolysis, about 95 per cent of the radioactivity was associated with the C_{15}-alcohols farnesol and nerolidol, but there was a striking difference in that the amount of nerolidol was now almost seven times greater than that of farnesol. Acid hydrolysis has also consistently resulted in the appearance of dimethylallyl alcohol, linalool, nerol (*cis*-geraniol), geraniol, a material with a retention volume on polyadipate columns of 1.85 times that of farnesol (corresponding to the C_{20}-tertiary alcohol geranyllinalool), and occasionally also a substance with a retention volume of 3.56 times that of farnesol.

Further analyses of the alcohol portions of the allyl pyrophosphates have been made after hydrolysis of these compounds with other phosphatases. In each instance the aqueous solution of the allyl pyrophosphates was extracted with petroleum ether before hydrolysis with the phosphatase. Samples of allyl pyrophosphate preparation No. 3 were also hydrolyzed

FORMULAE



(I)



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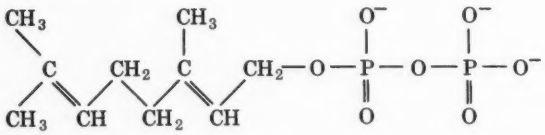

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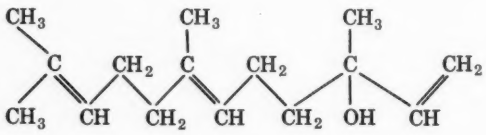

(IV)

with intestinal alkaline phosphatase and with the phosphatase present in liver microsomes (the latter in 0.1 M tris-HCl buffer pH 7.4; see below). With each of these two phosphatase preparations, the same alcohols were found as after hydrolysis with prostatic acid phosphatase. The farnesol-to-nerolidol ratio differed somewhat from one preparation to another, for the activity of nerolidol was only 1 to 2 per cent of the total after intestinal alkaline phosphatase hydrolysis, whereas after hydrolysis with microsomes, it was the same as after prostatic acid phosphatase.

A sample of allyl pyrophosphate preparation No. 1 was hydrolyzed at pH 8.3 with snake venom phosphatases for 24 hours, in which time only 75 per cent hydrolysis was achieved. Analysis of the alcohols so released again revealed the presence of dimethylallyl alcohol, geraniol, nerolidol, and farnesol. Farnesol plus nerolidol represented 80 per cent of the total radioactivity, and the amount of farnesol was six times that of nerolidol.

One further technique, suggested by Dr. J. W. Cornforth, was employed to identify the alcohols of the pyrophosphate esters and in particular to decide whether nerolidol was a genuine enzymic product or not. A portion of allyl pyrophosphate preparation No. 1, containing 7,000 cpm. of C^{14}, was freeze-dried and then refluxed for 75 minutes with 2 ml. of 0.78 M ethereal solution of $LiAlH_4$; a method used by Karrer and Jucker (27) for the fission of cetylphosphate. After decomposition of the $LiAlH_4$ by careful addition of water, the layer of ether was drawn off, and the aqueous phase extracted three times with small portions of ether. The combined ether extracts were washed three times with 2 ml. of water and then the ether dried with $MgSO_4$. A total of 4,000 cpm. of C^{14} were found in this ether extract. Gas-liquid radiochromatographic analysis revealed the presence of two hitherto unidentified components (? hydrocarbons) and of farnesol and nerolidol, the ratio of the amounts of the latter two being 5:1.

These data therefore indicate that under the conditions employed for the biosynthesis of allyl pyrophosphates, the major product obtained was farnesyl pyrophosphate (formula I). In preparation No. 3 this compound represented more than 85 per cent of the total C^{14}-activity of the reaction products. 3,3-Dimethylallyl pyrophosphate (formula II) and geranyl pyrophosphate (formula III) are also always present, but in much smaller amounts. A small amount of nerolidol (formula IV) was always found in the analyses of the alcohols released by methods other than acidification, but the farnesol-to-nerolidol ratio varied slightly from one method to another. The question of the existence of nerolidyl pyrophosphate as an enzymic product is considered under Discussion. The differences between the analyses of the alcohols released by acid hydrolysis and those released by enzymic hydrolysis are undoubtedly due to rearrangements occurring during acid hydrolysis, and will also be discussed later.

Stability of the Allyl Pyrophosphates. For a study of the stability of the allyl pyrophosphates at various pH's, identical samples (containing 1,000 cpm.) of allyl pyrophosphate preparation No. 3 were added to 0.1 M buffers (3 ml.) of various pH's. After standing at 22°C for 6 hours, the mixtures were made alkaline (pH 10) with aqueous KOH and were extracted (after addition of 2 mg. of a mixture of allylic alcohols) with petroleum ether. The C^{14}-counts found in the petroleum ether extract were expressed as a percentage of the total C^{14}-counts that were released by strong acid and were taken to represent the degree of cleavage of the allyl pyrophosphates. The pH ranges 2.0 to 4.0 were covered by potassium phthalate-HCl buffers, pH 4 to 6 by sodium acetate buffers, and pH 6 to 8 by potassium hydrogen phosphate buffers. Since over 85 per cent of the allyl pyrophosphate preparation No. 3 consisted of farnesyl pyrophosphate (Fig. 1*c*), the data shown in Figure 2 are thought to depict the properties of farnesyl pyrophosphate. Above pH 5.5

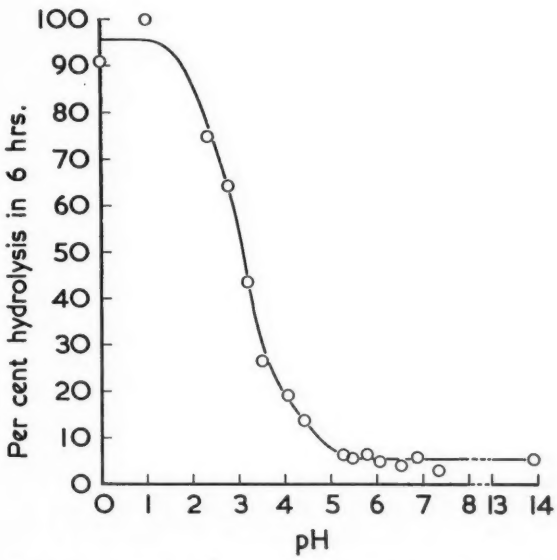

FIG. 2. Instability of farnesyl pyrophosphate as a function of pH. The percentage hydrolysis observed in 6 hours is plotted against the pH (see text for details of experimental procedure).

the compound is relatively stable, although it does hydrolyze very slowly even in alkaline solution. Below pH 5, however, farnesyl pyrophosphate hydrolyzes with a rate which varies inversely with pH. The implications of the sigmoid shape of the curve will be considered later. At any pH, hydrolysis at room temperature continues until it has gone to completion. Figure 3 is a plot of per cent hydrolysis as a function

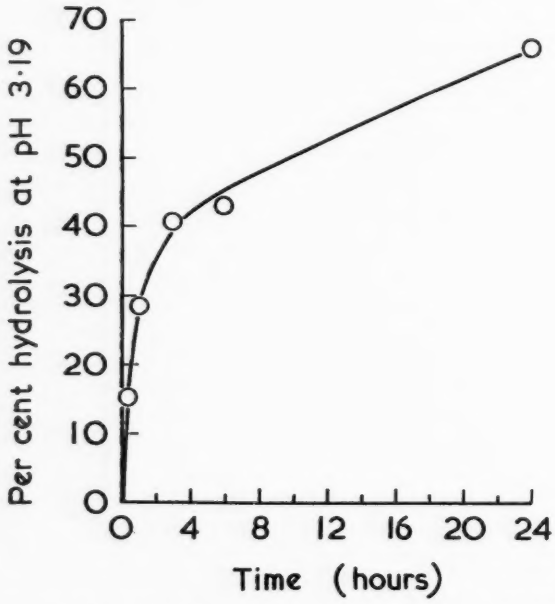

FIG. 3. Rate of hydrolysis of farnesyl pyrophosphate in 0.1 M phthalate buffer, pH 3.19, at 22°C.

of time at pH 3.19, a point near the center of the sigmoid curve in Figure 2. At pH 4, about 5 days are needed for complete hydrolysis.

Farnesyl pyrophosphate is fairly stable when heated in alkaline or neutral solution. Solutions of this compound in 0.1 N KOH were heated for 1 hour at 100°C, 80°C, and 60°C. This resulted in a hydrolysis of 15 per cent, 10 per cent, and 8 per cent, respectively. Heating a solution in 0.1 M potassium hydrogen phosphate buffer, pH 7.4 (measured at 22°C), at 100°C for 1 hour caused 28 per cent hydrolysis. When kept frozen at −20°C, however, farnesyl pyrophosphate was very stable (less than 2 per cent hydrolysis in 3 months).

Synthesis of Squalene from Farnesyl Pyrophosphate. The conversion of farnesyl pyrophosphate into squalene was studied in several experiments. Allyl pyrophosphate preparation No. 3 was used as substrate in all these experiments. The optimum reaction mixture for the anaerobic conversion of farnesyl pyrophosphate into squalene contained in a final volume of 1 ml. washed microsomes, 0.05 ml.; $MgCl_2$, 5 mM; TPNH or DPNH, 1 to 3 mM; nicotinamide 30 mM; NaF, 10 mM; potassium phosphate buffer, pH 7.4, 0.1 M; plasma albumin, 2 mg.; and C^{14}-farnesyl pyrophosphate, 0.03 to 0.1 μmole. This mixture will be referred to as the "complete squalene synthesizing system." Unless otherwise specified, TPNH was always

TABLE 3. COMPONENTS OF THE SQUALENE SYNTHESIZING SYSTEM OF RAT LIVER*

Washed microsomes	0.05 ml.
$MgCl_2$	5 mM
TPNH† or DPNH	1-3 mM
Nicotinamide	30 mM
NaF	10 mM
Potassium PO_4 buffer, pH 7.4	0.1 M
Plasma albumin	2 mg.
C^{14}-farnesyl pyrophosphate	0.03–0.1 μmole

* Complete anaerobic incubation mixture contained in 1.0 ml.
† Usually generated with G-6-P + glucose-6-phosphate dehydrogenase.

generated in the incubation mixture by the addition of TPN, G-6-P, and purified glucose-6-phosphate dehydrogenase. Nicotinamide was added in order to inhibit microsomal destruction of the pyridine nucleotides and sodium fluoride to inhibit a potent microsomal phosphatase which hydrolyzes the allyl pyrophosphates. Table 4 demonstrates that this phosphatase

TABLE 4. HYDROLYSIS OF ALLYL PYROPHOSPHATES BY RAT LIVER MICROSOMES*

Buffer	Additions	Hydrolysis
		per cent
Tris-HCl	None	7
Tris-HCl	Microsomes, 0.05 ml.	43
Phosphate	Microsomes, 0.05 ml.	16
Tris-HCl	Microsomes, 0.05 ml.; NaF 10 μmoles	11
Phosphate	Microsomes, 0.05 ml.; NaF 10 μmoles	8

* Each incubation contained in a final volume of 1 ml. 3700 cpm. of C^{14}-labeled allyl pyrophosphates (0.03 μmole of farnesyl pyrophosphate) 2 mg. bovine plasma albumin, 5 mM $MgCl_2$, 30 mM nicotinamide, and 0.1 M buffer pH 7.4. Incubations under N_2 at 37°C for 1 hr.

was strongly inhibited by the combination of phosphate buffer and fluoride. With the complete squalene synthesizing system containing TPNH, the yield of squalene from farnesyl pyrophosphate varied from 50 to 80 per cent. The squalene formed was identified by chromatography on alumina, followed by formation of the thiourea adduct.

The pyridine nucleotide requirements for this system are shown in Table 5. When these nucleotides were not added, or TPN alone was used, squalene was not formed. With TPNH, however, there was an excellent yield of squalene; with DPNH the yield was also good, but less than with TPNH.

TABLE 5. NEED FOR REDUCED PYRIDINE NUCLEOTIDES IN THE SYNTHESIS OF SQUALENE FROM FARNESYL PYROPHOSPHATE*

Addition	Squalene
	cpm.
None	70
TPN (1 μmole)	120
TPNH (1 μmole†)	2670
DPNH (1 μmole)	1900

* Each tube contained in 1 ml. the complete squalene synthesizing system (Table 3) without TPNH (or DPNH), and with 3300 cpm. (0.03 μmole) of C^{14}-farnesyl pyrophosphate. Incubations under N_2 for 1 hr. at 37°C.

† Generated from 1 μmole of TPN with 3 μmoles of G-6-P and purified glucose-6-phosphate dehydrogenase.

The need for the presence of a divalent cation was demonstrated by using microsomes which had been dialyzed for 2 hours against a large volume of 0.02 M phosphate buffer containing 1 mM EDTA. Purified TPNH was used in these experiments. The data given in Table 6 demonstrate that in the absence of a divalent cation, the yield of squalene was very small (less than 10 per cent). On the addition of either Mg^{++}, Mn^{++}, or Co^{++}, however, a good yield of squalene (55 to 60 per cent) was obtained. Nickel ions were able to satisfy partially the cation requirement, but Cu^{++}, and Ca^{++} ions were inhibitory. That this system requires a divalent cation could also be inferred from the fact that in the presence of an excess EDTA the synthesis of squalene was completely inhibited (Table 8).

The rate of squalene formation in this system is shown in Figure 4. These studies also provide the best comparison between the effectiveness of TPNH and DPNH, since identical concentrations of purified reduced pyridine nucleotide (2 μmoles per ml.) were used. It is apparent that the reaction shows very little specificity for either TPNH or DPNH.

TABLE 6. DIVALENT CATION REQUIREMENT FOR SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Addition	Squalene
	cpm.
None	372
Mg^{++} 5 μmoles	2320
Mn^{++} 5 μmoles	2220
Co^{++} 5 μmoles	2340
Ni^{++} 5 μmoles	735
Ca^{++} 5 μmoles	120
Zn^{++} 5 μmoles	87
Cu^{++} 5 μmoles	22

* Each tube contained in 1 ml. the complete squalene synthesizing system (Table 3) without $MgCl_2$ with 4000 cpm. (0.036 μmole) of C^{14}-farnesyl pyrophosphate, 1 μmole of TPNH, and 0.1 ml. of microsomes dialyzed against 1 mM EDTA. Incubations under N_2 for 1 hr. at 37°C.

Evidence has been obtained that only the farnesyl pyrophosphate present in the substrate solution of allyl pyrophosphates was used for squalene synthesis in these experiments.[1] The fact that up to 80 per cent yield of squalene could be obtained from a solution, more than 85 per cent of which was farnesyl pyrophos-

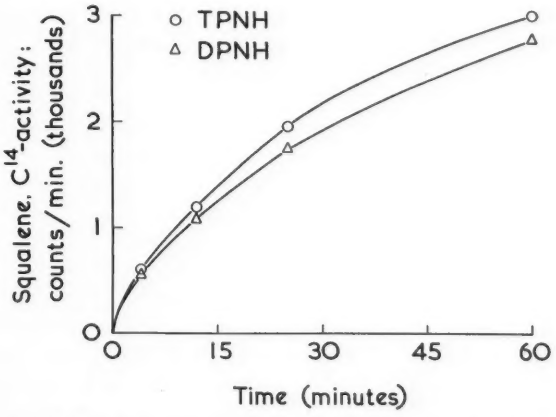

FIG. 4. Rate of squalene synthesis from farnesyl pyrophosphate. Aliquots (0.5 ml.) withdrawn at time intervals shown from 3 ml. complete anaerobic incubation mixtures (Table 3) containing 6000 cpm. of farnesyl pyrophosphate/ml., and 2 μmoles/ml. of either TPNH or DPNH.

[1] Nerolidyl pyrophosphate, if it were a true enzymic product in this solution (see Discussion), would probably have also participated in this reaction.

phate, indicates, of course, that most of the squalene must be synthesized from this compound. These data do not, however, establish that the small amounts of dimethylallyl pyrophosphate and geranyl pyrophosphate do not also participate in this reaction under the conditions employed. In order to investigate this question, the saponified incubation mixtures which had contained TPNH and DPNH in the experiment recorded in Table 5 (i.e., the incubations that gave a good yield of squalene) after extraction of the squalene were acidified, and the liberated alcohols extracted after neutralization. These alcohols, which represented the unused allyl pyrophosphates in these incubations, were analyzed by gas-liquid radiochromatography. The ratio of the amount of C_5- plus C_{10}-alcohols to that of the C_{15}-alcohols present was found to be 0.163. The value of this ratio for the original substrate solution (Fig. 1) was 0.057. These data therefore demonstrated a considerable enrichment of the shorter chain alcohol pyrophosphates in the solutions in which squalene synthesis took place. It can be inferred therefore that only the C_{15}-compounds were used in this reaction.

The Effect of pH on Squalene Synthesis from Farnesyl Pyrophosphate. The effect of pH on the synthesis of squalene from farnesyl pyrophosphate was studied in the pH range 6.0 to 8.6. This range was covered by potassium phosphate (6.0 to 7.4) and tris-HCl (7.4 to 8.6) buffers. Separate experiments were made with TPNH and DPNH. The results showed (Fig. 5) that the optimum pH was 7.4, although squalene synthesis proceeded well over the entire range studied. The explanation for the differences noted with TPNH and DPNH is obscure.

Effect of Soluble Enzymes. When dialyzed soluble enzymes were added to the complete squalene synthesizing system, the yield of squalene was consistently reduced, and under these conditions there occurred some conversion of the allyl pyrophosphates into carboxylic acids. The results shown in Table 7 are representative of this effect, and also demonstrate the fact that in the presence of soluble enzymes the difference between TPNH and DPNH in promoting squalene synthesis is considerably enhanced, with TPNH acting as a much more effective coenzyme for squalene formation. The nature of the carboxylic acids formed in this system, the mechanism of their formation, and their physiologic role are at present under investigation and will be the subject of a future report.

Inhibitor Studies. A variety of compounds was tested to ascertain whether they might inhibit the synthesis of squalene in this system. Table 8 contains

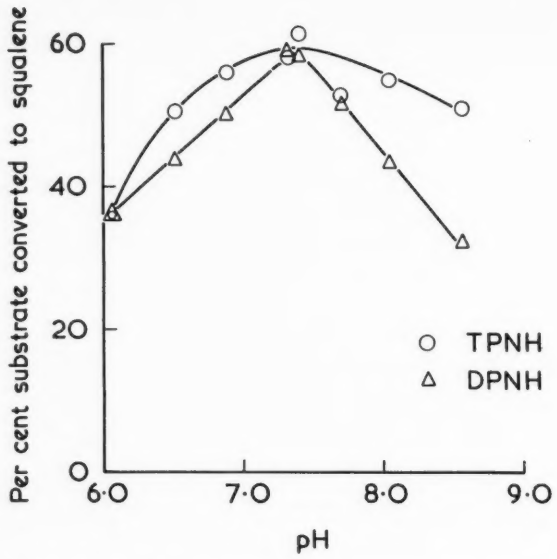

FIG. 5. Effect of pH on the synthesis of squalene from farnesyl pyrophosphate. One-hour complete anaerobic incubations at 37°C, in 0.1 M phosphate or tris buffer (see text).

the combined results of four sets of experiments. In each experiment a control, consisting of the complete squalene synthesizing mixture without inhibitor, was included. The per cent inhibition was calculated from the yield of squalene in the presence of the inhibitor, as compared to yield in the control for that experiment. The per cent inhibition is listed as "none" if the yield

TABLE 7. EFFECT OF SOLUBLE ENZYMES ON SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Additions	Squalene	Residual Allyl-PP	Carboxylic Acids
	cpm.	*cpm.*	*cpm.*
TPNH (1 μmole)†	6160	1400	160
DPNH (1 μmole)	4830	1570	120
F_{30}^{60}-enzymes (23 mg. protein) + TPNH†	4120	750	1500
F_{30}^{60}-enzymes (23 mg. protein + DPNH (1 μmole)	2230	720	2960

* Each tube contained 7500 cpm. of C^{14}-allyl pyrophosphates (7000 cpm., i.e., 0.064 μmole, of farnesyl pyrophosphate) and the complete squalene synthesizing system (Table 3) without TPNH or DPNH; the final volume was 1 ml. Incubations under N_2 for 1 hr. at 37°C.

† Generated from 1 μmole of TPN and 2 μmoles of G-6-P with glucose-6-phosphate dehydrogenase.

TABLE 8. INHIBITORS OF SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Additions†	Inhibition
μmoles	*per cent*
GSH (5)	none
BAL (10)	none
GSSG (10)	13
PCMB (0.01)	none
PCMB (0.1)	63
PCMB (1)	98
PCMB (2.5) + ascorbate (10)	100
PCMB (2.5) + GSH (5)	13
N-ethyl maleimide (1)	54
N-ethyl maleimide (3)	75
Iodoacetamide (0.2)	none
Iodoacetamide (2)	none
Iodoacetamide (10)	none
Na arsenite (0.5)	16
Na arsenite (3)	25
Na arsenite (3) + GSH (10)	none
Na arsenite (3) + BAL (10)	none
EDTA (20)	100
$Cu(NO_3)_2$ (1)	98
$CaCl_2$ (1)	61
$NiSO_4$ (1)	47
Zn acetate (1)	26
$FeSO_4$ (1)	15
$CoCl_2$ (1)	13
$MnCl_2$ (1)	none
KCN (1)	none

* Each tube contained in 1 ml. the complete squalene synthesizing system with 1 μmole of TPNH and 0.03 μmole of C^{14}-farnesyl pyrophosphate. In these experiments 1 μmole of TPN was first reduced with 3 μmoles of G-6-P and glucose-6-phosphate dehydrogenase; the components of the squalene synthesizing system and the inhibitors were added afterward (see also text). Incubations under N_2 for 1 hr. at 37°C.

† Numbers in parentheses indicate μmoles of inhibitors added.

of squalene in that tube was 90 per cent or more of the control yield. It is apparent from Table 8 that a variety of substances inhibit this system. PCMB is a very potent inhibitor, and its effects could be almost completely abolished by the addition of GSH. N-ethyl maleimide also inhibited the reaction, but the third SH-inhibitor, iodoacetamide, did not. Among the divalent cations tested (all in the presence of 5 mM Mg^{++}) Cu^{++} was the most potent inhibitor, squalene synthesis being completely abolished in its presence. Significant inhibition was also observed with Ca^{++}, Ni^{++}, and Zn^{++}. The two ions which were able to replace Mg^{++} in satisfying the cation requirement for squalene synthesis (Table 6) either exerted no (Mn^{++}) or only borderline (Co^{++}) inhibitory effects.

Free Alcohols. Studies were performed (together with Dr. J. Christophe) to ascertain whether the free allylic alcohols might be either phosphorylated and/or converted into squalene by rat liver enzymes. Experiments were made with C^{14}-alcohols released from the pyrophosphates by hydrolysis with prostatic phosphatase and with those obtained after acidification. Incubation mixtures were set up containing microsomes with or without dialyzed, unfractionated soluble enzymes (F_0^{100}-enzymes), ATP, and TPNH, in various combinations, and together with $MgCl_2$, nicotinamide, NaF, and phosphate buffer in the usual concentrations. After a 1-hour anaerobic incubation at 37°C, the solutions were analyzed for squalene, free alcohols, and allyl pyrophosphates. In no instance was more than a trace of radioactivity (less than 2 per cent of the total activity in all cases) found in either the squalene or the allyl pyrophosphate fractions. These results indicate that if phosphorylation of the free alcohols and/or squalene synthesis from the free alcohols (mainly farnesol) occurs at all, it is insignificantly small.

DISCUSSION

The results of the experiments reported here indicate that the reactions of squalene biosynthesis in rat liver are similar to those occurring in yeast cells. It has been demonstrated that in the yeast cell these reactions include the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, the condensation of these two compounds to give geranyl pyrophosphate and the condensation of geranyl pyrophosphate with isopentenyl pyrophosphate to yield farnesyl pyrophosphate (3, 6). The present findings, that a fraction of soluble enzymes from rat liver homogenates catalyzes the formation of farnesyl pyrophosphate from mevalonate or from 5-phosphomevalonate in excellent yield, and that dimethylallyl pyrophosphate and geranyl pyrophosphate are also always formed during this process, indicate that these same reactions occur in the liver cell. It has also been shown that iodoacetamide completely inhibited the synthesis of allyl pyrophosphates from 5-phosphomevalonate. Since iodoacetamide does not inhibit mevalonic (10) and phosphomevalonic kinase from liver,[2] it must inhibit either diphosphomevalonic decarboxylase or isopentenyl pyrophosphate isomerase. Lynen *et al.* (6) have shown that in the yeast system only the latter enzyme is inhibited by iodoacetamide.

[2] Hellig and Popják, unpublished observations.

It thus seems likely that it is this same enzyme which is inhibited in the liver system also.[3]

There can be no doubt that farnesyl pyrophosphate is the major (or only) sesquiterpenoid intermediate in squalene biosynthesis, since squalene can be obtained from it in yields as high as 80 per cent. In both the liver and the yeast cell the reactions involved in the formation of farnesyl pyrophosphate (and of the other allyl pyrophosphates) from mevalonate are catalyzed by enzymes found in the soluble fraction of the disrupted cells. The only cofactors needed for the synthesis of the allyl pyrophosphates from mevalonate or from 5-phosphomevalonate are ATP and a divalent cation. It was demonstrated in Bloch's laboratory (2, 3) that 3 moles of ATP are required for the formation of 1 mole of isopentenyl pyrophosphate from 1 mole of mevalonate, and both Chaykin *et al.* (2) and Lynen *et al.* (4) have shown that squalene synthesis can proceed from isopentenyl pyrophosphate without ATP. It follows, therefore, that in the soluble liver enzyme system the ATP is probably needed only for the formation of isopentenyl pyrophosphate and that the synthesis of geranyl and farnesyl pyrophosphates proceeds by the electrophilic condensation of dimethylallyl pyrophosphate and of geranyl pyrophosphate with isopentenyl pyrophosphate, as proposed by Lynen *et al.* (4), by Rilling and Bloch (28), and by Cornforth and Popják (29), and proved experimentally by Lynen and his colleagues (6).

The instability of the allyl pyrophosphates at acid pH as well as their reactivity in enzymic reactions is attributable to their chemical structure, as discussed by Cornforth (30). Allyl pyrophosphates, in common with other substances in which a potentially anionic group is in the α-position to a double bond, are electrophilic reagents and can be expected to lose the pyrophosphate ion rather readily. The electron deficient species $\left[\begin{array}{c} | \quad\quad\quad | \\ C \cdots CH \cdots C \\ | \quad\quad\quad | \end{array} \right]^+$ resulting from the elimination of the pyrophosphate ion, may be partially stabilized by resonance between two equivalent forms $\begin{array}{c} | \quad\quad | \\ C{=}CH{-}C^+ \\ | \quad\quad | \end{array}$ and $\begin{array}{c} | \quad\quad | \\ C^+{-}CH{=}C \\ | \quad\quad | \end{array}$. In the presence of water these two forms may yield, by the addition of a hydroxyl ion, either a primary or a tertiary alcohol. This is the mechanism of an allylic rearrangement, and is undoubtedly responsible for the conversion of a large part of farnesyl pyrophosphate to nerolidol during acid hydrolysis (detailed discussion in reference 31). The equilibria involved differ from one compound to another, for while most of the farnesyl pyrophosphate is converted to nerolidol, only a small fraction of the geranyl pyrophosphate is converted into the corresponding tertiary alcohol linalool. In addition, other rearrangements seem to occur during acid hydrolysis of the pyrophosphates, such as a small degree of *cis-trans* isomerization resulting in the appearance of some nerol from geranyl pyrophosphate. The allylic rearrangement of farnesyl pyrophosphate was also noted by Lynen *et al.* (6).

Evidence that it is the undissociated allylpyrophosphoric acid which is the unstable species is provided by the data shown in Figure 2. It will be noted that this plot of per cent hydrolysis in 6 hours against pH resembles a titration curve. Furthermore, the data shown in Figure 3 indicate that with longer periods of hydrolysis, this plot can be expected to move to the right, whereas with shorter time intervals, it will lie farther to the left (probably with associated minor changes in the shape of the curve). Now the value of pK_1 for the dissociation of a single hydrogen ion from farnesyl pyrophosphoric acid can be expected to be the same as that for pyrophosphoric acid, namely, 1.9. If it is indeed the undissociated acid which is the unstable species, it can be expected that with shorter and shorter periods of hydrolysis, the limiting position of the curve in Figure 2 will have pH 1.9 as its midpoint. At 6 hours the curve lies only one pH unit to the right. There is, of course, a good theoretical reason to expect the undissociated acid to be the unstable molecular species. The mechanism of elimination from an allyl pyrophosphate discussed above requires that the pyrophosphate moiety extract an electron from the organic portion of the molecule and leave as an anion. If the pyrophosphate portion of the molecule already bears a negative charge by virtue of the dissociation of one or more hydrogen ions, it will be much less likely to be able to extract still another negative charge (an electron) from the organic portion of the molecule.

Evidence for the correctness of these postulated mechanisms comes from the present demonstration of a quantitative relation between the amounts of inorganic pyrophosphate and of free alcohol liberated during acid hydrolysis of allyl pyrophosphates and from the observed allylic rearrangement of farnesyl pyrophosphate to nerolidol. Lynen *et al.* (4) demonstrated qualitatively (by paper chromatography) the appearance of inorganic pyrophosphate during acid hydrolysis of farnesyl-P^{32}-pyrophosphate biosynthesized from mevalonate and P^{32}-labeled ATP.

[3] Since this paper was submitted, it was found that in incubations of F_{30}^{60}-enzymes with mevalonate, ATP, Mg^{++}, and 5 mM iodoacetamide, isopentenyl pyrophosphate alone accumulated.

The data on the synthesis of squalene from farnesyl pyrophosphate demonstrate that the enzyme(s), which we propose to call the squalene synthetase system, is associated with the microsomes, and that the only cofactors necessary for this reaction are reduced pyridine nucleotides and a divalent cation (Mg^{++}, Mn^{++}, or Co^{++}). The reaction shows very little specificity for TPNH or DPNH, and in fact the differences shown in Figure 4 might be considered as due to differences in reduction potential of the two nucleotides. The squalene synthetase system of yeast was likewise found to be associated with cell particles and to need reduced pyridine nucleotides as coenzymes (4, 6). Spectrophotometric experiments (not reported here) have suggested a one-to-one stoichiometric relation between the amount of TPNH utilized and the amount of squalene formed. This conclusion is only provisional, however, because of the experimental difficulties encountered in measuring the allyl pyrophosphate dependent oxidation of small amounts of TPNH in the presence of microsomes.

The reductive step in squalene biosynthesis is somehow intimately connected with the tail-to-tail joining of two sesquiterpenoid intermediates, for in the absence of added TPNH or DPNH, all the C^{14} originally added as substrate can still be recovered as farnesyl pyrophosphate and free farnesol after 1 hour of incubation with washed microsomes. The possibility that a sulfhydryl group on the enzyme(s) is somehow involved in this reaction is suggested by some of the data presented in Table 8. Thus, PCMB and N-ethyl maleimide inhibited squalene synthesis from farnesyl pyrophosphate, and the PCMB inhibition could be reversed almost completely by addition of GSH. On the other hand, a third sulfhydryl inhibitor, iodoacetamide, was without effect on this reaction. The question of the participation of a sulfhydryl enzyme in this reaction hence cannot be clearly answered as yet, and is under further investigation.

The question of the existence of nerolidyl pyrophosphate as an enzymic product is of importance for the formulation of the detailed mechanism involved in the conversion of farnesyl pyrophosphate into squalene. Cornforth and Popják (29) have recently advanced an hypothesis for this mechanism which assumes the existence of an isomerase capable of transforming farnesyl pyrophosphate into nerolidyl pyrophosphate (11).

Condensation of 1 molecule of farnesyl pyrophosphate with 1 molecule of nerolidyl pyrophosphate (analogous to the condensation of dimethylallyl or geranyl pyrophosphate with isopentenyl pyrophosphate) then results in a triterpenoid compound which, by appropriate eliminations, stabilizes to dehydrosqualene, the reduction of which to squalene is presumed. It is stated, however, that the chemical hypothesis advanced does not require the existence of nerolidyl pyrophosphate per se, but rather that the same mechanism could apply for any nerolidyl derivative which would have properties similar to those expected of nerolidyl pyrophosphate. This hypothesis is in accord with all the known experimental facts of squalene biosynthesis, and it explains satisfactorily the findings of Rilling and Bloch (28), that during squalene biosynthesis from 5-D_2-mevalonate, 10 atoms of deuterium, out of a possible 12 atoms, were incorporated into squalene, and that the 2 atoms that disappeared were lost from the 2 central carbon atoms of squalene.

The results obtained in the present experiments unfortunately do not provide an unequivocal answer to the question of whether nerolidyl pyrophosphate exists as an enzymic reaction product or not. Given the instability of farnesyl pyrophosphate, and the facility with which this compound rearranges to nerolidol during nonenzymic hydrolysis, the small amounts of nerolidol observed after enzymic hydrolysis must be viewed with suspicion, as possibly arising from some slight nonenzymic hydrolysis. On the other hand, nerolidol has been observed in every case, no matter what method of enzymic hydrolysis was employed and despite all precautions against acid catalyzed hydrolysis and even after reductive cleavage with $LiAlH_4$. The fact that the percentage of nerolidol was small is virtually immaterial; Lynen *et al.* (6) reported, for example, that the equilibrium position of isopentenyl-pyrophosphate isomerase is such that an equilibrium mixture of the products contains 93 per cent dimethylallyl pyrophosphate and only 7 per cent of isopentenyl pyrophosphate. It is therefore felt that a definite answer to this question cannot as yet be given, and will depend on the development of a method for the separation of the several allyl pyrophosphates from one another. Only when nerolidyl pyrophosphate is separated by such a method, and the nerolidol shown to be optically active, will its existence as an enzymic reaction product be established conclusively. As mentioned above, however, uncertainty as to the existence of nerolidyl pyrophosphate need not materially affect the hypothesis of the mechanism of squalene synthesis given by Popják and Cornforth (31). The essential feature of this hypothesis is the condensation of farnesyl pyrophosphate with a nerolidyl derivative, and this latter could even be an enzyme-substrate

complex (or compound). Perhaps it is here that a sulfhydryl group participates in the process.

One other interesting feature of the liver enzyme system is the fact that when dialyzed soluble enzymes are added to the complete microsomal squalene synthesizing system, the yield of squalene from farnesyl pyrophosphate is reduced and conversion of the substrate to carboxylic acids occurs. The formation of "terpenoid" acids from mevalonate in liver enzyme incubations has been previously noted (32), *trans-trans*-farnesoic acid being one of the acids identified. It is clear now from experiments carried out in collaboration with Dr. J. Christophe (to be published) that these acids arise by the action of soluble liver enzymes on the free allylic alcohols liberated from the pyrophosphates by the microsomal phosphatases. Unpublished studies indicate that these acids inhibit squalene synthesis, and the possibility exists that this system is involved in some way in the physiological regulation of endogenous cholesterol synthesis.

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Immunochemical studies of organ and tumor lipids: VIII. comparison of human tumor and ox spleen cytosides*

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SUMMARY

Preparations of cytolipin H, a human tissue lipid hapten containing fatty acid, sphingosine, glucose, and galactose in equimolar proportions (cytoside), have been compared with preparations of similar composition isolated from ox spleen. The iodine number and the specific rotation of preparations derived from human tumor are significantly larger than those from ox spleen. Results of other chemical analyses (based on the carbohydrate and sphingosine portions of the molecule) as well as immunochemical reactions are indistinguishable for both.

Repeated injection into rabbits of cell fractions or homogenates of foreign tissue provokes the formation of antibodies that frequently react with lipids extracted from the tissue. With human tissues this phenomenon is more readily seen with fractions derived from cancerous tissues than with similar fractions from normal organs. With many antisera, several independent antigen-antibody systems have been found to contribute to the over-all measurement (1). However, one substance, cytolipin H, and its antibody appear to be most frequently responsible for a major part of the reactions observed *in vitro* (1). Cytolipin H, a glycolipid isolated recently from human epidermoid carcinoma (H.Ep.3) grown in conditioned rats (2), is a water-insoluble substance containing four residues in equimolar proportions: fatty acid, lipid base (sphingosine), glucose, and galactose.

A molecule with this composition and similar physical properties was reported to be present in ox spleen by Klenk and Rennkamp in 1942 (3). In connection with the isolation of spleen gangliosides, they encountered a fraction which they called "cerebroside with two hexose residues" since it contained fatty acid, sphingosine, and two different hexoses. From studies based on fermentability, rotation, and reducing power of the water-soluble hydrolytic products, and assuming the carbohydrates to be galactose and glucose, the value of 1.25 was found for the ratio of galactose to glucose.

It is the purpose of this report to present a comparison of the chemical, physical, and immunological properties of these similar glycolipids found in human tumor and in ox spleen. Small but significant differences were found in specific rotation and iodine number. On the other hand, the preparations were indistinguishable by other chemical analyses and by immunological criteria.

It may be anticipated that the finding of biological (haptenic) activity in a molecule of this type will require some simplification in nomenclature in dealing with structure-activity relationships. For this reason the term "cytoside" has been introduced (4) for a molecule composed of ceramide (fatty acid amide of sphingosine) linked to two monosaccharide residues, in strict analogy with the useful term "cerebroside" describing a molecule in which ceramide is linked to one monosaccharide. We shall accordingly refer in this

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paper to preparations of human tumor and ox spleen cytosides.

METHODS

Cytoside Preparations. Two preparations of cytolipin H were isolated as described (2) from human epidermoid carcinoma (H.Ep.3) grown in rats. Two preparations of ox spleen cytoside were isolated similarly, the essential steps involving extraction of crude lipids from ox spleen, removal of water-soluble substances, fractionation on silicic acid, fractionation on Florisil,[1] and recrystallization from pyridine-acetone (2). From 10 kg. of tissue, 50 mg. of pure cytoside was obtained. Paper chromatography of the water-soluble, acid hydrolysis products of all preparations showed the presence of only two substances, these migrating at the same rates as glucose and galactose.

Chemical Analyses. For total hexose determination, samples of 100 to 150 μg. of cytoside were hydrolyzed for 2 hours in sealed tubes with 0.5 ml. of 3 N aqueous hydrochloric acid in a bath at 100°C. The tubes were opened, and the contents evaporated to dryness over sodium hydroxide in a vacuum desiccator. The residues were dissolved in 0.80 ml. of water, and a portion (0.60 ml.) taken for analysis. The analytical method was based on color formation with o-aminodiphenyl (5), using equimolar mixtures of glucose and galactose as standards.

Anthrone analysis was performed according to the method of Radin *et al.* (6). In this method glucose produces about 40 per cent more color than galactose, and a slightly larger difference (47 per cent) is observed with the corresponding cerebrosides. The color yield of cytoside is close to the predicted value for a molecule with one residue of each hexose.

For the determination with glucose oxidase, samples were hydrolyzed and evaporated to dryness as in the analysis for total hexose, and the residue was dissolved in 0.40 ml. of water. To 0.20 ml. of this solution was added 0.05 ml. of an aqueous solution of glucose oxidase in 0.05 M phosphate buffer (vial of Worthington "Glucostat" dissolved in water and made up to 10 ml.). After 2 hours at room temperature (20° to 25°C), 0.35 ml. of water was added, and analysis for residual hexose was made with o-aminodiphenyl (5), using galactose as the standard. Results are expressed as per cent of total hexose present as glucose.

Kjeldahl N was determined according to the modification of Tompkins and Kirk (7). Samples of approximately 500 μg. of cytoside were analyzed, yielding about 7 to 8 μg. of N.

Ninhydrin color was determined as follows: Samples of 100 μg. of cytoside were hydrolyzed in 13 $\times$ 150 mm. tubes (covered by "tear drop"-shaped glass caps) for 2 hours at 90°C with 0.5 ml. of 1.2 N hydrochloric acid in 90 per cent ethanol (8). Evaporation was reduced to a minimum by inserting the tubes into the bath only to the level of liquid in the tubes. Samples were carefully evaporated to dryness in a vacuum desiccator over sodium hydroxide and then analyzed without transfer by the method of Lea and Rhodes (9). The molar color yield of sphingolipids is about 75 per cent of that of leucine. It was essential that every trace of free hydrochloric acid be removed to obtain correct analytical values. With ethanol, this step frequently required prolonged periods of drying. This analytical method worked very well with cerebroside and sphingomyelin preparations isolated from animal tissues. It has not given satisfactory results with synthetic derivatives of dihydrosphingosine.

The iodine number on samples of 1.5 to 2 mg. of cytoside was determined by a micromodification of the Yasuda method (10). Specific rotation was measured in a Keston polarimeter at 589 mμ. at a concentration of about 2 per cent in pyridine.

Immunological Analysis. Isofixation curves (11) were obtained for the four cytoside preparations by determining 2 points in the zone of antibody excess and 3 points in the zone of antigen excess, using a rabbit antiserum (No. 161) prepared by injecting a particulate fraction of human cervix carcinoma (1). The complement level was six 50 per cent units (0.0085 ml. of guinea pig serum). Auxiliary lipid (100 parts by weight of a mixture of equal quantities of lecithin and cholesterol) was combined with cytoside in organic solvent (1). The solvent was evaporated in a stream of nitrogen. The dry residue was taken up in 0.1 ml. of methanol and then 0.9 ml. of saline was added. Dilutions were made with saline. Further details of the complement fixation test appear in preceding publications (1, 11, 12).

RESULTS

Chemical Analysis. The chemical analysis of two preparations of cytolipin H and two preparations of ox spleen cytoside are shown in Table 1. Values are referred to weight units rather than mole units because the fatty acid residues present in these preparations are mixed.[2] Although small differences are observed

[1] Floridin Company, Tallahassee, Fla.

[2] V. P. Skipski and M. M. Rapport, unpublished studies.

TABLE 1. CHEMICAL ANALYSIS OF HUMAN TUMOR AND OX SPLEEN CYTOSIDES

Cytoside Preparation	Hexose	N	Anthrone As*	Ninhydrin As†	Glucose	Iodine Number	$[\alpha]_D$‡
	per cent	*per cent*			*per cent hexose*		*degrees*
Human tumor I	36.8	1.38	125	135	49.7	32.0	−10.8
Human tumor II	36.2	1.50	128	137	51.0	35.4	− 9.1
Ox spleen I	34.0	1.36	130	137	48.9	22.6	− 7.3
Ox spleen II	34.5	1.43	125	139	52.9	27.0	− 6.9

* Absorbancy of solution (1%, 1 cm., 625 mμ.).
† Absorbancy of solution (1%, 1 cm., 575 mμ., aqueous methylcellosolve).
‡ In pyridine. See text for temperature and concentration.

among the four samples, five of these methods do not permit the differentiation of cytoside preparations of human tumor from those of ox spleen. For purposes of reference, it may perhaps be of value to record that a sample of phrenosine with a nitrogen content of 1.68 per cent gave a specific anthrone absorbance of 64 and a specific ninhydrin absorbance of 165. The small differences in total hexose are not reflected in the anthrone values, and are very likely the result of small variations in both extent of hydrolysis of the cytoside sample and stability of the hydrolytic products.

Within experimental error, all four preparations contain 50 per cent of the total hexose as glucose. It would appear, therefore, that the value of 1.25 for the ratio of galactose to glucose reported by Klenk and Rennkamp (3) is incorrect. The most probable explanation for their result is that on hydrolysis, galactose is released more readily than glucose (2); incomplete hydrolysis would thus produce more free galactose than free glucose.

With regard to iodine number, both preparations of cytolipin H (human tumor cytoside) show the presence of more than one double bond per mole. One double bond would give an iodine number of about 26. The ox spleen cytosides are more saturated, and since the value for one of the preparations is less than 26, the lipid base may in part be dihydrosphingosine. These differences in unsaturation are largely reflections of differences in the fatty acid residues of these particular preparations, and possible variations in the saturation of the sphingosine residue cannot contribute more than a small part.

The concentrations, temperatures, and observed absorbancies for the specific rotations recorded in column 8 are as follows: human tumor I, 2.07 per cent, 24°, 0.036; human tumor II, 1.77 per cent, 27°, 0.025; ox spleen I, 2.15 per cent, 27°, 0.024; ox spleen II, 1.83 per cent, 27°, 0.024. The value for the specific rotation

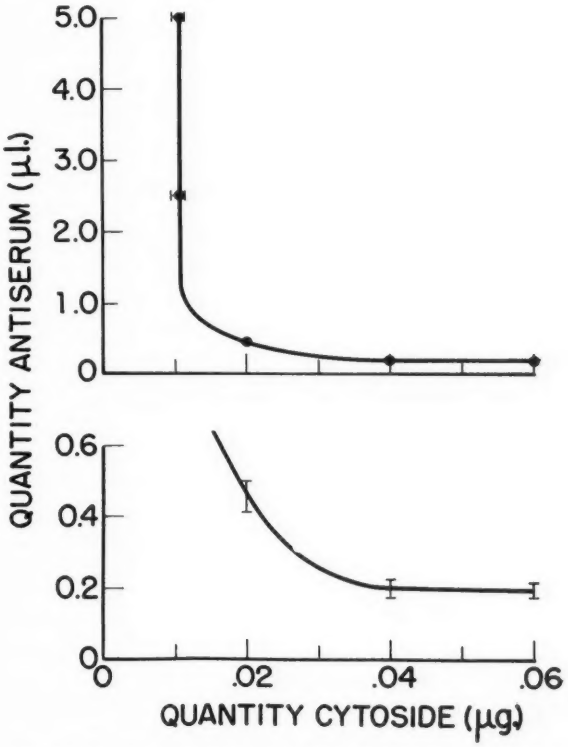

FIG. 1. Isofixation curve drawn through points representing averages of values obtained with two preparations of cytolipin H and two of ox spleen cytosides. The range of the four values at each point is shown by the short horizontal line in the upper figure (region of antibody excess) and by the short vertical line in the lower figure (region of antigen excess shown with the ordinate magnified fivefold). See text for details of complement-fixation test.

at 20° recorded by Klenk and Rennkamp for their preparation of ox spleen cytoside is — 6.81° (3). Although the rotations of all preparations are in the same direction and of the same order of magnitude, the human tumor cytosides have appreciably larger rotations than the ox spleen cytosides. Much of this difference may, as before, reflect differences in the fatty acid residues. None of these four cytoside preparations contained phosphorus (less than 0.02 per cent). None was chromogenic with ninhydrin before hydrolysis.

Immunological Characterization. Figure 1 shows the quantities of each cytoside preparation that react with various quantities of antiserum to produce the same end point (50 per cent hemolysis) in the presence of a constant quantity of guinea pig serum (complement) and a constant number of sensitized sheep red cells. Each point in the upper isofixation curve represents the average of the four values obtained with the four different cytoside preparations. The range of values for the two points in the region of antibody excess is shown by the short horizontal lines drawn through each point in the upper curve. In order to show this variation for the three points in the region of antigen excess, the lower curve is presented. It is the same as the upper curve except that the ordinate axis is magnified fivefold. The range of values is indicated by the short vertical lines through each point. It is evident that the four cytoside preparations are indistinguishable in this complement fixation test.

DISCUSSION

This study shows that cytoside preparations from human tumor and ox spleen have very similar chemical properties and are serologically identical in the complement fixation test with specific antibody. Since these preparations each contain a range of fatty acid residues (one that differs between individual preparations because of solubility selection during purification), it seems probable that an explanation for the observed differences will be found in the fatty acid portion of the molecule. In any case, the immunological method is not sensitive to this difference, suggesting that the specificity for the interaction with antibody resides principally in the carbohydrate portion of the structure. An excellent opportunity should thus be at hand for determining the configuration of this structure using the method of hapten inhibition, and such studies are in progress.

The finding that ox spleen and human tumor cytosides exhibit identical immunological behavior is of great importance for the further study of lipid haptens of human tissues. One reason is that it offers a solution to the problem imposed by limitations in the availability of human tissues. Despite the low concentrations of cytoside and complications in isolation arising from the presence in spleen of such closely related compounds as ceramide, cerebroside, and ganglioside, this tissue is an easily accessible, unlimited source of supply. A second reason is that it confirms the fact that hapten activity is an integral property of a molecule composed of fatty acid, sphingosine, glucose, and galactose. The observation that a high degree of biological activity is associated with a natural product and is retained through a limited number of purification steps does not exclude the possibility that an impurity in the final preparation may be the active principle. The most rigorous proof requires unequivocal synthesis of the compound in question. However, the fact that, as an immediate consequence of the isolation of cytolipin H, our attention was directed to a previously unsuspected source of material, and that this improbable result has been fully realized, goes far in establishing the validity of the original observation. It thereby offers a promise that an additional discipline, namely, immunochemistry, can be brought to bear on the study of one of the most interesting and complex classes of cellular substances, the sphingolipids.

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The question of biohydrogenation of fatty acids*

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SUMMARY

Rats were fed methyl palmitoleate-1-C^{14} and the palmitic acid was isolated from their organs and depot fat. Degradation studies revealed that very little activity found its way into the palmitic acid by hydrogenation of the palmitoleate. The major route of carbon from the unsaturated to the saturated acids is through acetate.

In 1937, Rittenberg and Schoenheimer (1) carried out an experiment designed to assess the amount of hydrogenation of unsaturated fatty acids occurring in mammalian tissues. Unsaturated fatty acids labeled with deuterium were obtained from mice that had been fed highly labeled saturated acids. The ethyl esters of these acids were fed to two mice, and the concentration of deuterium in their saturated and unsaturated fatty acids and body water were determined. It was found in both cases that the per cent excess deuterium in the saturated acids (0.047 and 0.025) was about 20 per cent of that in the unsaturated acids (0.25 and 0.10) and about equal to that in the body water. The conclusion drawn was that since previous experiments had shown that the saturated fatty acids synthesized in the presence of deuterium-enriched water ultimately achieve a deuterium content one-quarter to one-third that of body water, the excess deuterium in the saturated acids in this experiment could have been derived only by hydrogenation of the body unsaturated acids. This conclusion may not have been justified since the fed unsaturated fatty acids, which had an excess deuterium content of 1.02 atom per cent, were undoubtedly partially broken down following absorption and prior to complete equilibration with the total body unsaturated acids. There was thus a richer source of deuterium-containing substrate available for synthesis of the saturated fatty acids.

There has been some indirect evidence, based on analysis of animal depot and tissue fats, that hydrogenation of unsaturated fatty acids may occur (2 to 5). It is possible, however, to explain these data as well on other bases.

Recently considerable evidence has accumulated indicating that in the mammal fatty acid hydrogenation is not an important reaction. Steinberg *et al.* (6) fed carboxy-labeled linolenic acid to rats and isolated the linoleic acid from their depot and organ fats. Although the fed acid had an activity of 2.16×10^7 disintegrations per second per mmole, that of the linoleic acid was only 92, an activity which might be expected from the small contribution by partial equilibration of the first two carbons with the active acetate derived by oxidation of the linolenic acid. Mead and Howton (7), in a similar study with γ-linolenic acid, found that although the activity of the fed acid was 3.84×10^7 disintegrations per second per mmole, that of the isolated linoleic acid was only 176, while that of oleic acid was only 141, both of these activities being considerably smaller than those of the saturated acids. There is thus little evidence that hydrogenation in this sense (conversion of polyunsaturated to di- and monounsaturated acids) occurs.

Bernhard *et al.* (8) investigated this problem by feeding randomly labeled oleic, linoleic, and linolenic acids to rats and comparing the activities of the saturated acids isolated from their liver and carcass fats. In each case the activity of the stearic acid was about the same as that of the palmitic, and somewhat less than that of the cholesterol isolated from the same source. The authors reasoned that if appreciable hy-

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drogenation had occurred, the stearic acid should be considerably more active than the palmitic, which must obtain its activity largely by total synthesis. The cholesterol activity was taken as a measure of the acetate pool, from which the palmitate would be synthesized. However, these experiments were not designed to detect a low order of hydrogenation and consequently could not prove or disprove its existence. Moreover, the use of the cholesterol activity as a measure of the activity of the acetate available for fatty acid synthesis may not have been justified.

It was thus evident that there was a need for an experiment which would give a definite answer to this question. Such an answer could be obtained by the use of carboxy-labeled palmitoleic acid, since hydrogenation of this acid in the animal body would give palmitic acid with an excess of activity in the carboxy group. It has been shown in several laboratories (9, 10, 11) that in palmitic acid synthesized from acetate-1-C^{14} by well-nourished animals, the activity in all the odd-carbon atoms (including the carboxyl) is approximately equal. If the palmitic acid derived from such an experiment contained an excess of activity on the carboxy group, hydrogenation would have been demonstrated and its quantitative significance could be assessed.

Consequently, palmitoleic acid was isolated from macadamia nuts, which contain approximately 20 per cent of this acid. This was converted to the carboxy-labeled acid by known methods and was fed to normal rats. The results of these experiments permit the evaluation of the hydrogenation process in these animals under the stated conditions.

METHODS

All melting points are corrected. Gas chromatography was performed with either (*a*) an Aerograph Model A-100-C (Wilkens Instrument and Research Co., Inc.), using a 10-foot, ¼-inch column for analytical results or a 5-foot, ½-inch column for the preparative experiments, both packed with Craig Polyester-succinate (1,4-butanediol-succinate polymer); or (*b*) a Wheelco Model 10 (Barber-Colman Company) with a 6-foot, 6-mm. (I.D.) column of 80 to 100 mesh chromosorb siliconized by treatment with dimethyldichlorosilane (12). Reported *trans*-contents of olefinic substances, based on infrared absorption at 10.3 μ., are considered to be within 5 per cent of actual values.

Ultimate analyses were performed by Dr. A. Elek (Elek Micro Analytical Laboratories, Los Angeles) and the infrared absorption analyses by Mr. G. V. Alexander and Mr. Paul Kratz of this laboratory.

Preparation of Palmitoleic Acid. Shelled macadamia nuts (500 g.)[1] were alternately ground in a mortar and extracted with pentane, evaporation of which gave 376 g. (75 per cent) of a light yellow oil. This was saponified with 2 l. of 8 per cent alocholic KOH under nitrogen and the fatty acids (351 g.) were isolated in the usual manner and converted to the methyl esters. A sample analyzed on the gas chromatograph revealed a composition closely approximating that found in the literature (13). The remainder was fractionally distilled through a 2.5 x 100 cm. heated Vigreaux column at 0.9 mm. pressure and 135°-145°C. Three 50-ml. fractions were collected. The first showed approximately 87 per cent palmitoleate with 6 and 7 per cent, respectively, of myristate and a C_{18} acid and a trace of palmitate. Crystallization of this fraction (43.2 g.) from 2.5 l. of methanol at −60°C gave 12.6 g. of a soluble fraction containing approximately 93 per cent methyl palmitoleate and no detectable amounts of saturated esters. This material was saponified with 9 per cent KOH in methanol-water at room temperature overnight.

A sample purified further by distillation at 80 μ. and a bath temperature of 165°-170°C gave material with m.p. −0.9° to + 0.3°C and $n_D^{25.0}$ 1.4565. These values are in excellent agreement with the reported m.p. of 0°-0.5°C (14) and a linear plot of refractive index versus temperature made from published data (14, 15). The *p*-phenylphenacyl ester melted at 55.7°-57.1°C (56.5°-57°C [14]).[2]

threo-9,10-Dibromohexadecanoic Acid. Ten g. of palmitoleic acid in CCl_4 was brominated at 0°C as detailed for oleic acid (17) to give (without a low temperature crystallization) a quantitative yield of the dibromo acid as a viscous brownish oil. A sample of this crude material was purified by chromatography on a silicic acid column to obtain a colorless viscous oil of $n_D^{25.0}$ 1.4937. The *p*-phenylphenacyl ester was obtained after silicic acid chromatography and five crystallizations from 95 per cent ethanol as clusters of very thin colorless blades melting at 54.5°-55.1°C. Analysis calculated for $C_{30}H_{40}Br_2O_3$: C, 59.2; H, 6.6; Br, 26.3 per cent. Found: C, 59.0; H, 6.8; Br, 26.2 per cent.

threo-1,8,9-Tribromopentadecane. From 11.55 g.

[1] The macadamia nuts were obtained as a generous gift through R. H. Purdy, of the Pacific Vegetable Oil Co., Richmond, Calif.

[2] Baudart (16) prepared the *p*-bromophenacyl ester, m.p. 40°-40.5°C, which Boughton *et al.* (14) mistakenly compared with their *p*-phenylphenacyl ester.

(0.0237 mole) of silver dibromohexadecanoate (prepared in 93.3 per cent yield from the acid) reaction with bromine in dry CCl_4 as described previously (17) produced 5.35 g. (0.0119 mole or 50.2 per cent) tribromopentadecane; 0.75 g. (0.00107 mole or 9.0 per cent) presumably of 8,9-dibromopentadecyl 9,10-dibromohexadecanoate, the Simonini-type ester expected as a by-product of the Borodin reaction (it was not further characterized); 2.52 g. (0.00609 mole or 25.7 per cent) of recovered dibromohexadecanoic acid; and 0.80 g. of unidentified material, together accounting for 95 per cent of the starting dibromohexadecanoate.

Rechromatography of the crude tribromopentadecane on silicic acid produced a colorless, viscous oil of $n_D^{28.5}$ 1.5076 and $d^{28.5}$ 1.4025 g. per ml. Assay of this material by gas chromatography indicated that it contained only 70 to 80 per cent of tribromopentadecane.

cis-1-Bromo-8-Pentadecene. This compound was prepared from 4.49 g. (0.0100 mole) of once-chromatographed 1,8,9-tribromopentadecane in benzene by reaction with 4.0 g. (2.0061 mole) granular zinc preactivated with HBr under absolute ethanol; after refluxing for a total of 35 minutes, the mixture was worked up as described elsewhere (17) to give, after a preliminary distillation, 2.34 g. (81.0 per cent yield) of a colorless oil. By gas chromatography, this material was 93 per cent bromopentadecene. Using gas chromatography on a preparative scale, followed by silicic acid chromatography to remove any volatile products from the stationary liquid phase and vacuum distillation to remove solvent, there were obtained 127 mg. of a colorless mobile oil of $n_D^{30.0}$ 1.4676 and $d^{30.0}$ 1.0148 g. per ml. The molar refraction calculated from these constants was 79.19 and may be compared with a theoretical value of 79.766. Analysis by gas chromatography showed this product to be better than 99 per cent bromopentadecene, with a small amount of lower homologue and a trace of what is probably the saturated bromopentadecane. The *trans* content was estimated to be 5.0 per cent. Analysis calculated for $C_{15}H_{29}Br$: C, 62.3; H, 10.1; Br, 27.6 per cent. Found: C, 62.2; H, 10.0; Br, 27.5 per cent.

The sample of pentadecenyl bromide used in the next step contained, by gas chromatography, 95 per cent pentadecenyl bromide and approximately 5 per cent of a 17-carbon bromide; no saturated 15-carbon bromide was detectable. The *trans* content was 9.8 per cent.

Methyl Palmitoleate-1-C^{14}. The preparation and carbonation of the Grignard reagent from 0.856 g. (2.96 mmole) of pentadecenyl bromide and 3.10 mmole of carbon dioxide containing 1.927 mc. C^{14} were carried out as described previously for heptadecenyl bromide (17). The 0.2207 g. (0.822 mmole or 27.7 per cent from pentadecenyl bromide) of methyl palmitoleate thus obtained was shown by gas chromatographic analysis to be 97 per cent pure, with 3 per cent C_{18} ester and no trace of palmitate. In the second of the two feeding experiments performed (see below), however, the palmitoleic acid was purified further by reversed phase chromatography with removal of the portion of the fraction which might contain any palmitic acid impurity. The activity of the methyl palmitoleate was 1.04×10^5 d.p.s. per mg.[3] or 1.09×10^5 d.p.s. per mg. for the free acid. For the first feeding experiment, 111 mg. of this acid was diluted to 804 mg. with inactive methyl palmitoleate.

Animal Experiments. Two experiments were performed using slightly different conditions. In experiment I, 755 mg. of the diluted methyl palmitoleate (1.1×10^7 d.p.s. total) was administered orally to three nonfasted male rats weighing a total of 786 g. on a normal commercial diet.

In experiment II, two male rats of similar weight were placed for 10 days on the same diet with additional 30 per cent of corn oil, before administration of 405 mg. of methyl palmitoleate (5.6×10^6 d.p.s. total). The rationale behind this treatment was, first, to inhibit fatty acid synthesis, thus proportionally increasing the possible effect of hydrogenation and, second, to provide an excess of unsaturated fat with the hope that a hydrogenation mechanism might be emphasized. An additional change involved the further purification of the fed palmitoleic acid, as described above.

In both experiments the rats were killed after 4 hours and the organs and depot fat were removed, frozen, and lyophilized. The 4-hour time was chosen since it is sufficient to allow complete absorption of the small amounts of oil administered and represents a compromise between maximum incorporation of activity into the various fatty acids and extensive catabolism to carbon dioxide. In experiment I, from 13.5 g. of dried tissues was obtained, following extraction with 4:1 methylal:methanol, 3.53 g. of fat with an activity of 63.6 d.p.s. per mg., saponification of which gave 2.61 g. of fatty acids with an activity of 85.0 d.p.s. per mg. Similarly, from experiment II was obtained

[3] Counting was performed with the single-channel room temperature liquid scintillation counter described by Hodgson *et al.* (18). At 1150 volts, sensitivity was 77.0 ± 0.2 per cent. An external standard was used for preliminary counts and an internal standard (40.7 d.p.s. of ring-labeled toluene) for all final counts to correct for self-absorption. Samples were counted in 3-dram vials dissolved in 5 ml. of toluene containing 5 g. phenylbiphenyloxadiazole and 0.10 g. 1,4-bis-2-(phenyloxazolyl)-benzene per liter.

20 g. of dried tissues from which was derived 8.8 g. of fatty acids with an activity of 5.4 d.p.s. per mg.

Preparation of Palmitic Acid. The total fatty acids from experiment I were crystallized from acetone at −30°C, giving 845 mg. of saturated acids with an activity of 20.0 d.p.s. per mg. A portion of the saturated acid mixture (331.5 mg.) was separated on the reversed phase column of Howard and Martin (19) as described previously (20), giving 233.5 mg. of palmitic acid, representing 82.5 per cent of the saturated acids and 26.5 per cent of the total fatty acids. Crystallization of this acid from acetonitrile gave 198.4 mg. of palmitic acid, which, when chromatographed as the methyl ester, showed no trace of any other substance. It had an activity of 8.42 d.p.s. per mg. palmitic acid.

Similarly, from experiment II was obtained 1.7 g. of saturated fatty acids with an activity of 0.32 d.p.s. per mg. and, following reversed phase chromatographic separation and crystallization of 319 mg. of this mixture, 233 mg. of pure palmitic acid with an activity of 0.35 d.p.s. per mg.

In an exactly similar manner, palmitic acid was derived from the saturated fatty acids of rats which had been fed carboxy-labeled γ-linolenic acid (7). This acid had an activity of 1.87 d.p.s. per mg. and served as a control for the palmitic acid derived from methyl palmitoleate.

Degradation of Palmitic Acid. Degradation was carried out by the method of Dauben *et al.* (10) with the modifications discussed previously (21). Only the carboxy-carbon, as benzoic acid, and the remaining pentadecanoic acid were counted.[4]

TABLE 1. ACTIVITIES OF FATTY ACIDS AND DEGRADATION PRODUCTS FROM METHYL PALMITOLEATE-1-C^{14}

Fraction		
	d.p.s./mg.	*d.p.s./mmole*
Palmitoleate Fed		
palmitoleic acid (prep'd)	1.09×10^5	2.78×10^7
Experiment I		
palmitoleic (fed)	1.44×10^4	3.66×10^6
total fatty acids	85.0	
saturated fatty acids	20.0	
palmitic acid	8.42	2158.9
benzoic acid	6.08	742.4*
(C_1 of palmitic acid)		
pentadecanoic acid	6.64	1621.8 (231.7)†
(C_2 thru C_{16} of palmitic acid)		
Experiment II		
palmitoleic acid (fed)	1.38×10^4	3.50×10^6
total fatty acids	5.42	
saturated fatty acids	0.32	
palmitic acid	0.22	56.9
benzoic acid	0.066	9.0*
(C_1 of palmitic acid)		
pentadecanoic acid	0.13	32.7 (4.7)†
(C_2 thru C_{16} of palmitic acid)		
γ-Linolenate Fed		
palmitic acid	1.87	479.5
benzoic acid	0.58	71.1*
(C_1 of palmitic acid)		
pentadecanoic acid	2.01	491.2 (70.2)†
(C_2 thru C_{16} of palmitic acid)		

* Since the total activity of the benzoic acid resides in the carboxy carbon, d.p.s./mmole = d.p.s./milliatom carbon.

† In parenthesis, d.p.s./milliatom active carbon.

Since palmitic acid formed from carboxy-labeled acetate has its activity in the odd carbons (even carbons for pentadecanoic acid) the activity per active carbon is $\frac{\text{d.p.s./mmole}}{7}$.

RESULTS AND DISCUSSION

In Table 1 are listed the activities of the various acids and their degradation products. It can be seen that for the palmitic acid formed by condensation of carboxy-labeled acetate derived from γ-linolenic acid, the ratio of the activity of carboxy carbon to the activity of average active carbon is 1.01. For the palmitoleic-fed rats, however, the ratio is 3.21 for experiment I and 1.92 for experiment II.

The activity of the palmitic acid isolated in these experiments is derived from several sources. The first is by direct hydrogenation of the fed palmitoleic acid. Since this activity would be in the carboxy group alone while that of the palmitic acid totally synthesized from the acetyl coenzyme A derived by degradation of the palmitoleic acid would be equally distributed in the odd carbons, its relative magnitude may be obtained by subtraction of eight times the activity per active carbon of the pentadecanoic acid from the activity of the whole (Table 1). In this manner it can be estimated that in experiment I, 21.5 per cent of the palmitic acid activity was derived by hydrogenation of palmitoleic acid while 78.5 per cent was the result of total synthesis. Similarly, from experiment II, 10.5 per cent of the activity was from hydrogenation and 89.5 per cent from total synthesis. Since in the γ-linolenate-fed animals, in which palmitic acid was totally synthesized from acetate, the carboxy carbon activity was equal to that of the average active carbon, this preliminary conclusion appears justified. However, it should be borne in mind that although both types of active palmitic acid (by hydrogenation or total synthesis) are equally diluted by inactive palmitic acid from other sources, the totally synthesized palmitic acid suffers a further dilution of a different sort. The acetyl coenzyme A derived by degradation of the palmitoleic acid is diluted by inactive coenzyme A from

[4] See footnote 3.

other sources and suffers losses to other synthetic processes. This dilution cannot be accurately measured since the pool of coenzyme A actually used for palmitic acid synthesis is probably different from that used for other acetylations. However, its result is to decrease the observed importance of the total synthesis pathway. Thus the estimates of its importance given below are probably minimal.

Although these figures seem to indicate that hydrogenation of the fed palmitoleic acid did indeed occur and that a definite fraction of the palmitic acid of the animal body was formed in this manner, they do not permit a direct calculation of the importance of this process in the general metabolism of fatty acids. This can be roughly estimated from the data obtained above with several approximations. Although the total fatty acids of the bodies of the rats were not isolated, it can be assumed that the sample used was representative of the whole. For experiment I, therefore, 140 g. of fat (18 per cent of body weight) (22) with 63.6 d.p.s. per mg. gives a total of 9×10^6 d.p.s. The same value is obtained if the calculation is based on total fatty acids and fatty acid activity. Therefore some 80 per cent of the total ingested activity is present in the total body fatty acids 4 hours after ingestion. Since in this experiment palmitic acid was found to be about 26 per cent of the total fatty acids, it may be estimated that 3×10^5 d.p.s. are present in the palmitic acid. If 21.5 per cent of this figure derives from hydrogenation, about 6.5×10^4 d.p.s. came from this reaction. Thus a maximum of about 0.6 per cent of the administered activity as palmitoleate appears in palmitate as the result of hydrogenation.

Whether this figure, taken at 4 hours, can be carried any further to give some idea of the total process on a continuous basis is doubtful. It may be instructive, however, to compare it with the conversion of γ-linolenic acid to arachidonic acid at the same time and under the same conditions. From the activities of the fed and isolated acids in this experiment (7), it can be estimated that 15 per cent of the fed γ-linolenate is present in arachidonic acid 4 hours after feeding. Thus it can be seen that in comparison with a process such as this (involving dehydrogenation and addition of acetate), the hydrogenation reaction is relatively minor.

Experiment II suffers from the very low activity recovered in the palmitic acid. It is evident that the dietary manipulations in this experiment were all too successful and that very little palmitic acid was synthesized from the acetate derived by degradation of the fed palmitoleic acid. It is equally evident that very little activity found its way into palmitic acid by any other route. The specific activity of the palmitic acid is very low and the excess activity in the carboxy group is even smaller than in experiment I. It is thus possible that some of the apparent evidence for hydrogenation in experiment I may have been the result of an immeasurably small but very active contamination of the fed palmitoleic acid by palmitic acid. Thus, for experiment II, the proportion of palmitic acid derived by hydrogenation of fed palmitoleate is considerably smaller than for experiment I, and a similar estimate of the amount of palmitic acid formed by hydrogenation of palmitoleate indicates a maximum of 0.01 per cent of the administered activity at 4 hours.

Although these studies serve to minimize the importance of biohydrogenation of unsaturated fatty acids as a major metabolic pathway, they do not disprove its existence. A definite portion of the active palmitic acid obtained from animals fed active palmitoleate was apparently derived by this route. This amount of hydrogenation would probably have escaped detection in the experiments of Bernhard *et al.* (8) and could have come to light only in experiments such as the above.

Another question not answered by the present experiments is whether the hydrogenation observed might have occurred in the intestine as a result of bacterial action. This will have to be answered before any further idea on the importance of biohydrogenation can be gained.

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Composition of molecular distillates of corn oil: isolation and identification of sterol esters*

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SUMMARY

Molecular distillates of corn oil consisting of the three most volatile 1.7 per cent cuts and the next most volatile 4.9 per cent cut obtained from 150 kg. of refined oil have been subjected to simple solvent and chromatographic fractionation. These fractions represented about 10 per cent of the original oil and some 50 per cent of its total unsaponifiable material. About 74 per cent of this unsaponifiable matter was sterol, of which 75 per cent was free, the rest combined—mainly as the long-chain fatty acid esters. The remainder of the analyzed unsaponifiable matter consisted of hydrocarbons (3.3 per cent), tocopherols (15.0 per cent), material more polar than free sterols (5.0 per cent), and various other uncharacterized fractions of differing polarity (2.7 per cent). The sterol ester fraction was made up largely of the linoleates, oleates, and palmitates of beta- and gamma-sitosterols and the triterpenoid alpha-sitosterols. Indications were obtained for the presence of tocopherol esters in corn oil. Among the glyceride esters the mono- and diglycerides of the major corn oil fatty acids were recognized representing, respectively, 0.05 and 0.52 per cent of the original oil. The refined corn oil was estimated to contain about 0.08 per cent acidic material of which more than 90 per cent appeared in the first most volatile 1.7 per cent cut. It contained mainly oleic and linoleic acids in about the same proportion as they occur in the corn oil.

Work performed in this laboratory (1, 2, 3) has demonstrated that some substance in the more volatile molecular distillates of corn oil, which are rich in unsaponifiable matter, accounts at least in part for the hypocholesterolemic effect of this foodstuff. Grande *et al.* (4) have reached a similar conclusion concerning the activity of the unsaponifiable matter of this oil. Of the compounds reported to occur in this fraction, alpha-tocopherol was shown to have no effect, whereas a preparation of beta-sitosterol, known to be grossly impure, did elicit a hypocholesterolemic response (1). This effect, first described by Peterson (5) in chicks and confirmed by Pollak (6) in man, has been demonstrated in many other laboratories (7), though a certain degree of variation has also been reported. This variation has been interpreted by some investigators (8, 9) as a result of a possible differential response on the part of the individual toward beta-sitosterol, a variation in the purity of the sterol preparation, or both. The possibility remains that plant sterols other than beta-sitosterol or indeed that some other type of compound associated with these substances may be primarily responsible for the hypocholesterolemic activity residing in the unsaponifiable matter of corn oil and its volatile molecular distillates.

In order to identify the agent or agents responsible for this hypocholesterolemic effect, extensive qualitative and quantitative analytical investigations of the unsaponifiable matter of corn oil enriched in the more volatile components have been undertaken. The availability of sufficient quantities of molecular distillates of this oil, provided through the courtesy of the Distillation Products Industries, of Rochester, New York, has greatly facilitated this study. In addition, the utilization of such distillates in these analyses has permitted an investigation of the naturally occurring but little-known plant sterol esters, thus yielding information impossible to obtain when working with the original oil or its alkaline hydrolysates. The following report describes methods found to be suitable for the fractionation of the above distillates and the isolation and preliminary characterization of several sterol and tocopherol esters as well as mono- and diglycerides, none of which have been hitherto reported to occur in corn oil.

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MATERIAL AND METHODS

The distillation constants and certain other characteristics of these distillates and the original oil are given in Table 1. All reagents and solvents employed

TABLE 1. DISTILLATION CONSTANTS* AND CERTAIN OTHER CHARACTERISTICS OF CORN OIL DISTILLATES†

Fraction	Temperature	Pressure	Original Oil	Color	State at Room Temperature
			Original Distillation		
		μ.	*per cent*		
1st Cut	227°–230°C	13–15	10	orange	solids in liquid
2d Cut	228°–232°C	7–9	10	yellow	liquid
3d Cut	230°C	10	30	yellow	liquid
Residue	(not distilled)		50	yellow	liquid
			Redistillation of 1st Cut		
1 D	235°C	6	1.7	yellow-orange	solid
2 D	240°C	4	1.7	orange	solids in liquid
3 D	240°C	4	1.7	amber	liquid
4 D	245°C	4	4.9	yellow	liquid

* The distillation constants were recorded by Distillation Products Industries, Rochester, N. Y.

† The original oil (Mazola) had the following characteristics: specific gravity (25°C) 0.9190, smoke point (°F) 465, saponification equivalent 191, A. O. M. stability at 98°C (hr.) 19, iodine value 125, phospholipids 0.003%, tocopherols about 0.1%, unsaponifiable matter 1.55%, sitosterols 1%. The fatty acid composition was: myristic 0.2, palmitic 9.9, stearic 2.9, saturated above C_{18} 0.2, hexadecenoic 0.5, oleic 30.1, linoleic 56.2, and linolenic 0, all as per cent of total. (D. M. Rathmann, personal communication to J. M. R. Beveridge.)

were reagent grade chemicals and, unless otherwise indicated, were used without further purification.

Solvent Fractionation of the Distillates. The acidic and the neutral components were separated by extracting an ethereal solution of the distillate with 0.05 N KOH. Recoveries of the acidic fraction plus neutral fat were approximately 98 per cent. The bulk of the free sterol was removed from the 1 D distillate by crystallization from ethyl acetate and methanol.

Fractionation of Neutral Lipids. The method was essentially a combination of those described by Fillerup and Mead (10) and Barron and Hanahan (11). It was validated by performing test runs with a number of reference compounds, such as paraffin, squalene, octadecyl palmitate, alpha-tocopheryl palmitate, beta-sitosteryl palmitate, triolein, octadecanol, alpha-tocopherol, lanosterol, beta-sitosterol, ergosterol, diolein, mono-olein, and 1,18-octadecanediol.

Fractionation of Sterol Esters. The sterol ester mixtures isolated by adsorption chromatography from the various distillates were resolved into a number of bands using the reversed phase paper partition chromatography method described earlier (12). In order to obtain sufficient amounts of material for saponification and paper chromatographic analysis of the hydrolysis products, separations were performed on full sheets of double thickness Whatman No. 3 filter paper.

Fractionation of Sterols. The reversed phase partition chromatography system of Sulser and Hoegl (13) was modified. Either an aqueous 40 per cent butyric or 88 per cent acetic acid solution equilibrated with paraffin oil served as the mobile phase. Whatman No. 1 filter paper was used and the chromatograms developed by the ascending technique. The sterol mixtures were run as bands, as described for the sterol esters. The method was checked with purified beta- and gamma-sitosterol.

Fractionation of Fatty Acids. For the separation of the unsaturated fatty acids, the reversed phase partition chromatography method of Schlenk *et al.* (14) was used. Both saturated and unsaturated fatty acids were simultaneously separated and semiquantitatively estimated by the method of Inouye and Noda (15). The reliability of both techniques was verified with mixtures of known fatty acids.

Characterization of Glyceride and Sterol Esters. The distillates or any fraction thereof were saponified with a solution of 8 per cent (w/v) KOH in aqueous methanol (95 per cent), using a ratio of 3 g. of KOH to 1 g. of lipid. Samples requiring less than 25 ml. of alkali solution were diluted to 25 ml. with benzene. The unsaponifiable matter and the fatty acids were recovered in the usual way. The aqueous fraction was used for the assay of glycerol (16). The sterols were either converted into digitonides (17) and assayed for fast- and slow-acting components (18) or were chromatographed on paper. The fatty acids were separated and identified by chromatography.

The total and alpha-monoglycerides were estimated by the periodate oxidation according to the method of Martin (19).

The iodine numbers were determined by the micromethod of Yasuda (20). Optical rotations were taken on 2 per cent solutions in chloroform in a Hilger Model M 412 polarimeter. Ultraviolet spectra were recorded on absolute ethanol solutions on a Beckman Model DK 2 ratio recording spectrophotometer. Infrared spectra were determined on a Perkin-Elmer Infracord spectrophotometer equiped with sodium chloride optics. Qualitative colorimetric tests for ketosteroids,

TABLE 2. APPROXIMATE DISTRIBUTION OF UNSAPONIFIABLE MATTER DURING MOLECULAR DISTILLATION OF REFINED CORN OIL

Fraction	Original Oil	Total Unsaponifiable Matter		Sterols			Tocopherols	Hydrocarbons
	per cent	*per cent distillate*	*per cent original unsap.*	*per cent distillate*			*per cent distillate*	*per cent distillate*
				total	*free*	*bound*		
	Original Distillation*							
Original oil	100	1.65	100	1.1	—	—	0.12†	0.02†
1st cut	10	8.05†	48.7	5.95†	4.39†	1.56†	1.2†	0.25†
2d cut	10	2.5	15.1	2.2	—	—	—	—
3d cut	30	1.0	18.2	0.9	—	—	—	—
Residue	50	0.6	18.1	0.4	—	—	—	—
	Redistillation of 1st Cut							
1st cut	10	8.05†	48.7	5.95†	4.39†	1.56†	1.2†	0.25†
1 D	1.7	31	31.9	22.5	19.5	3	7.0	1.3
2 D	1.7	10	10.2	7.15	3.65	3.5	0.3	0.05
3 D	1.7	4.2	4.3	3.5	1.0	2.5	0.02	0.01
4 D	4.9	0.76	2.2	0.65	trace	0.65	0.01	0.0

* Wherever a dash is shown, the determination was not performed.
† Estimated from analyses on fractions 1 D–4 D.

vitamin A, tocopherols, amino acids, and carbohydrates were made by the Zimmermann, Carr-Price, Emmerie-Engel, ninhydrin, and Molisch reactions, respectively. Phosphorus was determined by the method of Beveridge and Johnson (21) and nitrogen by nesslerization.

RESULTS AND DISCUSSION

Preliminary Characterization of Distillates. From the physical appearance of the distillates (Table 1), it was obvious that the first and the second most volatile 1.7 per cent cuts contained large quantities of free sterol that had crystallized out. Digitonin precipitation of the free sterol before and after saponification of portions of the distillates and gravimetric determination of the amounts of digitonides obtained indicated that the distillates 1 D, 2 D, 3 D, and 4 D contained about 22.5, 7.15, 3.5, and 0.65 per cent of total sterol, respectively, of which 13.5, 49.0, more than 71, and more than 95 per cent, respectively, occurred in the combined form (Table 2). Since all the known corn oil sterols had been reported (22) to form readily insoluble digitonides, this method was considered a valid estimate of the free and esterified sterol content.

With the digitonides on hand, an attempt was made to estimate the content of the fast-acting sterols by determining the modified Liebermann-Burchard color reaction time curves. Though some of the digitonides partially decomposed during drying and did not permit a fully satisfactory examination, it was noted that in none of the distillates were there significant amounts of fast-acting sterols. The optical density time curves rose gradually, showing no peak or shoulder after a 90-second reaction time, reaching a maximum after about 30 minutes and superimposing to a considerable extent with the curve obtained for a purified beta-sitosterol sample when adjusted for differences in concentration.

Determination of the ultraviolet absorption spectrum of the 1 D distillate showed a maximum at 294 mμ. and a large shoulder at 285 mμ. with a minimum at about 267 mμ. The location of the maximum and minimum and the general shape of the curve (23, 24) indicated that the distillate contained large quantities of tocopherol. Since esterification had been known (24) to shift the absorption maximum to about 286 mμ., the shoulder observed at this wave length was taken as an indication of the possible presence of tocopherol esters. This belief was supported by the

TABLE 3. APPROXIMATE DISTRIBUTION OF SOME SAPONIFIABLE LIPID COMPONENTS DURING MOLECULAR DISTILLATION OF REFINED CORN OIL

Fraction	Original Oil	Free Fatty Acids	Monoglycerides	Diglycerides	Triglycerides	Unknown Material
	per cent	*per cent distillate*				
	Original Distillation*					
Original oil	100	0.08†	0.05†	0.52†	97†	0.37†
1st cut	10	0.84†	0.49†	5.22†	80.66†	3.7†
2d cut	10	—	—	—	95	—
3d cut	30	—	—	—	98	—
Residue	50	—	—	—	99	—
	Redistillation of 1st Cut					
1st cut	10	0.84†	0.49†	5.22†	80.66†	3.7†
1 D	1.7	4.7	2.5	16.03	35	7.5
2 D	1.7	0.2	0.4	10.70	70	5.30
3 D	1.7	0.02	0.01	0.51	87	5.76
4 D	4.9	0.00	0.00	0.01	98	1.23

* Wherever a dash is shown, the determination was not performed.
† Estimated from analyses on fractions 1 D–4 D.

observation that an alkaline hydrolysis destroyed the shoulder with a concomitant increase in the maximum and decrease in the minimum absorption. The 2 D, 3 D, and 4 D distillates showed two maxima each at about 270 mμ. and 280 mμ., as did the original oil, superficially resembling the absorption curve for a delta-5,7-diene sterol. On saponification, however, none of this ultraviolet absorbing material appeared in the unsaponifiable matter, and consequently could not be attributed to ergosterol as was done by Heilbron *et al.* (25). The report of these workers claiming the presence of ergosterol in corn oil on the basis of ultraviolet light absorption has not been confirmed by an actual isolation of this sterol from corn oil or its unsaponifiable matter. The apparent absence of any significant amounts of fast-acting sterol from these distillates makes it unlikely that ergosterol is present in corn oil. Using an extinction coefficient $E^{1\%}_{1\ cm.}$ of 90 for a mixture of the corn oil tocopherols in ethanol solution at 294 mμ., the total tocopherol concentration in the pooled distillates was estimated at about 1 per cent. If all the tocopherol had distilled over into the first 10 per cent of the original distillation, this would represent a concentration of about 100 mg. of tocopherol per 100 g. of the refined oil, a figure well within the range of values recorded in the literature (26).

Determination of the neutral equivalent of the distillates by titration of suitable oil samples in chloroform with methanolic KOH and assuming an average molecular weight of 280 for the fatty acids of corn oil indicated that the 1 D distillate contained about 4 per cent of free fatty acids. Only traces of acidic material were detected in the other distillates (Table 3).

In addition, the distillates were analyzed for nitrogen and phosphorus by methods capable of detecting one part per million. While no phosphorus could be detected in any of the distillates, there were about 5 parts per million of nitrogen detected in the most volatile fraction, which, however, could not be associated with any of the fractions recovered from the silicic acid column, when spot checks on the eluted lipids were made. The fractions gave negative reactions for carbohydrates in the Molisch test.

Simple Solvent Fractionation of Distillate 1 D. During the initial characterization of the various distillates it was noticed that the 1 D distillate contained significant amounts of acidic material. Removal of this material by extraction with a mild alkali provided an easy access to it and considerably simplified the subsequent adsorption chromatographic fractionation. The small amounts of free fatty acids left in the other

distillates overlapped with the triglycerides when chromatographed on the silicic acid. The amount of the acidic material extracted from 1 D amounted to about 5 per cent of the distillate and was in fairly good agreement with the estimate of the free fatty acid content of 1 D obtained from the neutral equivalent determination.

The adsorption chromatographic fractionation of the neutral lipid of distillate 1 D was further facilitated by a preliminary removal of the bulk of the free sterol. Ethyl acetate-methanol recrystallization of 1 D removed approximately 17 per cent of crystalline material, part of which (0.5 per cent of 1 D) was a solid hydrocarbon (m.p. 50°C). The rest of the crystalline material consisted of a mixture of free sterols (m.p. 136°-138°C, α_D — 31°). Further solvent fractionation of the neutral 1 D lipid into material soluble and insoluble in absolute methanol, partition between methanol and ligroin, and low temperature crystallization from acetone were tried. These techniques produced a great variety of fractions but failed to yield any significant separation or noticeable simplification of the subsequent chromatography.

Adsorption Chromatography of Unsaponifiable Matter. Silicic acid chromatography permitted the fractionation of the unsaponifiable matter from the various distillates into several main groups of compounds. The elution pattern obtained with the 1 D distillate is represented in Figure 1; the approximate composition of the fractions is shown in Table 4. Essentially the same elution patterns were obtained with the unsaponifiable fractions of other distillates, except for diminished amounts of the more volatile substances. The results of these analyses of the various unsaponifiable fractions from the four distillates are summarized in Table 2. It may be noted that the figures obtained for the total sterol are in good agreement with those arrived at during the preliminary characterization of the various distillates by gravimetric digitonide determinations. The figures for total tocopherol are somewhat higher, but this was to be expected, as the spectrophotometric measurements made on the unsaponified distillates failed to correct for the lower extinction of the tocopherol ester at the wave length of analysis.

About 100 mg. of an unsaturated hydrocarbon, at least part of which was squalene, as indicated by the formation of a hydrochloride of correct melting point and approximately correct iodine number, was isolated from this unsaponifiable fraction. It corresponds to the amount present in about 31 g. of distillate 1 D. Assuming that all the unsaturated hydrocarbon was

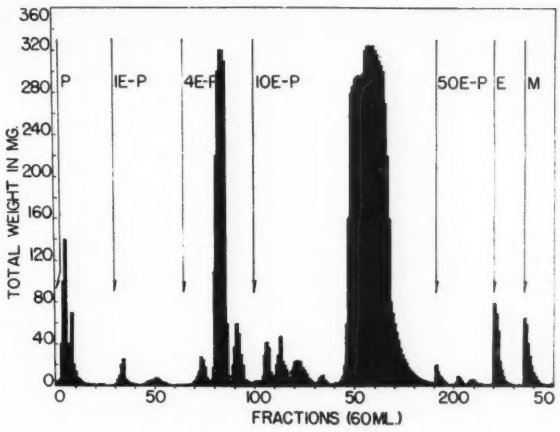

FIG. 1. Chromatography on silicic acid of the unsaponifiable matter from corn oil distillate 1 D. Solvents were petroleum ether (P), diethyl ether (E), and methanol (M). The composition of the various fractions is recorded in Table 4.

squalene and that all squalene distilled over into the 1 D fraction, this amount would fall within the range of squalene concentrations reported by Fitelson (27) for corn and other seed oils. However, the absorption spectrum of this unsaturated hydrocarbon indicated that there was also some carotene present. It appears that the quantities of both squalene and carotene isolated represented only part of these substances originally present in the corn oil, as it is known that at least carotene, because of its high unsaturation, distills irregularly (28) under the molecular distillation conditions and is polymerized (29) and peroxidized in the process. Further degradation of the more unsaturated components of these distillates took place during the analytical manipulation (fraction VIII, Table 4).

Adsorption Chromatography of Neutral Lipids. The preliminary removal of the bulk of the free sterol and all the acidic material was both convenient and desirable for a successful adsorption chromatographic fractionation of the residue. Though with carefully adjusted solvent polarity it has been possible to separate free fatty acids from triglycerides (30) and free sterols, usually the acids are eluted together with the triglycerides (11). The sterol removal rendered the residue much more soluble in the solvents of low polarity, making the initial adsorption of the material on the adsorbent column possible from reasonable quantities of petroleum. It also permitted a rather clean-cut resolution of the free sterol and the diglyceride peaks. The nature of the fractionation pattern of the lipid remaining in 1 D after the removal of the free fatty acids and most of the free sterols is graphically depicted in Figure 2. The solvents and their vol-

TABLE 4. COMPOSITION OF FRACTIONS OBTAINED ON SILICIC ACID CHROMATOGRAPHY OF THE UNSAPONIFIABLE MATTER FROM CORN OIL DISTILLATE 1 D*

Fractions in Order of Elution	Eluting Solvent	Volume of Eluting Solvent	Components	Fraction Weight	Original Material	Remarks
		ml.		*mg.*	*per cent*	
I	Petroleum ether	1800	Saturated hydrocarbons	300	3.0	Waxy solid, m.p. 30°–50°C, iodine number 0.7
			Squalene and carotene	100	1.0	Yellow oil, λ_{max} 440 mμ., HCl, m.p. 122°C, iodine number 359
II	1% ethyl ether in petroleum ether	2000	Ketones	50	0.5	White semisolid, slightly Zimmermann positive, iodine number 55
III	4% ethyl ether in petroleum ether	2000	Tocopherols, alcohols, C_4 Me sterols	1960	20.0	Yellow oil, λ_{max} 294 mμ., λ_{min} 267 mμ., Emmerie-Engel positive
IV	10% ethyl ether in petroleum ether	2000	Sterols	450	4.5	Cryst. solid, L-B positive, m.p. 126°–132°C, α_D −10 to −24°
V	10% ethyl ether in petroleum ether	3000	Sterols	6500	66.0	Cryst. solid, L-B positive, m.p. 136°–138°C, α_D −30°
VI	50% ethyl ether in petroleum ether	1800	Sterols	35	0.3	Cryst. solid, L-B positive, m.p. 138°–140°C, α_D −20°
VII	Absolute ethyl ether	1000	Unidentified alcohols	300	3.0	Amber-colored oil, L-B negative, α_D 0.6°
VIII	10% methanol in ethyl ether	1000	Unknown	300	3.0	Dark red glass, probably peroxidized squalene and carotene degradation products, L-B negative, α_D 0°

* 9.85 g. of lipid was applied to 600 g. of silicic acid. The total recovery was 9.99 g. (101%). See Figure 1 for the elution pattern.

umes used together with the composition of the major fractions and certain remarks are recorded in Table 5. Similar elution patterns were obtained on silicic acid chromatography of the other distillates, except that there was less of the more volatile material present, with a consequent increase in the proportion of the triglyceride. Table 3 summarizes the data obtained for the major saponifiable lipid components present in the various distillates.

The fractions obtained by this means, although apparently clearly separated, were found to be contaminated with preceding or succeeding fractions, or both. A similar observation has been reported by Lovern (31), who, working with the lipids of haddock flesh, obtained quite sharp peaks on silicic acid chromatography, but subsequently found that these components did not necessarily represent pure fractions. For further study, such as the sterol and sterol ester separations described below, or for a quantitative assay, the fractions had to be rechromatographed, preferably with the same solvent sequence as that used in the original experiment.

In view of the results presented, it appears that most of the sterol in corn oil exists in the free state. However, the distillates analyzed contain only a part of the total free and combined sterol present in the original oil, since significant amounts of the unsaponifiable material, much of which is sterol, have been left behind in the second and third most volatile cuts and in the residue from the original distillation (Table 2). Because of the greater volatility of the free sterol (32), the unsaponifiable matter (51.3 per cent of original unsaponifiable matter) left behind in the bulk of the oil during the original distillation, is likely to contain a much greater proportion of the combined sterol form. The elimination of only about 50 per cent of the total unsaponifiable matter of corn oil into the first most volatile 10 per cent fraction during the

original distillation should be contrasted with an elimination of about 95 per cent of the total unsaponifiable material of soybean oil into the corresponding fraction during a similar distillation (33). Assuming that sterols make up most of the unsaponifiable matter in both oils, and knowing that the sterol esters are considerably less volatile than the free sterols, it would appear that a large sterol ester concentration, such as observed for the corn oil, might not be the property of all plant oils.

Estimation of alpha-mono- and the total monoglyceride content before and after perchloric acid isomerization (19) indicated that practically all (97 per cent) of the monoglyceride was present as the 1-monoglyceride. An estimation of the proportion of the major fatty acids, e.g., palmitic, oleic, and linoleic, present in the various glycerides and the original oil by paper chromatography (15), indicated an approximate ratio of 10, 40, and 50, respectively (cf. also 34). An approximately similar ratio for these fatty acids was also observed for the sterol ester fraction, but the presence of other fatty acids was indicated.

Naturally occurring plant sterol esters have been isolated only rarely (35), usually by accident, since the small amounts present in common lipid extracts may be lost in any large-scale systematic analyses. This is easily understood since techniques apparently suitable for obtaining lipid preparations enriched with sterol esters, such as molecular distillation (28, 36) and preferential enzymatic destruction of the triglyceride (37, 38) and their effective fractionation by adsorption chromatography (10, 11, 30), have been exploited only recently. The recent publication of the paper partition chromatographic methods for the separation of beta- and gamma-sitosterol mixtures (13) and the long-chain fatty acid esters of sterols (12, 39) has now permitted an examination of this class of compounds.

TABLE 5. Composition of Fractions Obtained on Silicic Acid Chromatography of the Neutral Lipids (Low in Free Sterols) from Corn Oil Distillate 1 D*

Fractions in Order of Elution	Eluting Solvent	Volume of Eluting Solvent	Components	Fraction Weight	Original Material	Remarks
		ml.		*mg.*	*per cent*	
I	Petroleum ether	1800	Hydrocarbons	210	2.0	Waxy solid, m.p. 30°–50°C, plus a yellow oil, iodine number 387
II	1% ethyl ether in petroleum ether	2500	Aliphatic alcohol, tocopherol esters and sterol esters	1380	13.0	Light yellow oil, L-B positive, λ_{max} 285 mμ., λ_{min} 260 mμ., F.A. 41%, sterol 45%, tocopherol 11%, unacc. 3%
III	4% ethyl ether in petroleum ether	2600	Triglycerides and tocopherols, alcohols, C_4 Me sterols	3720	35.0	Light yellow oil, λ_{max} 294 mμ., λ_{min} 267 mμ., 20% tocopherol, fatty acid/glycerol molar ratio 3.2
IV	10% ethyl ether in petroleum ether	5400	Residual sterols and tocopherols	2660	25.0	Solid, L-B positive, 1% tocopherol; sterol recryst. m.p. 132°C, α_D −28°
V	25% ethyl ether in petroleum ether	3600	Diglycerides	1700	16.0	Colorless oil, slightly L-B positive, fatty acid/glycerol molar ratio 2
VI	50% ethyl ether in petroleum ether	1800	Unknown alcohols	75	0.8	White oil, fatty acid/nonlipid alcohol molar ratio 1.5
VII	Absolute ethyl ether	1800	Monoglycerides	320	3.0	Colorless oil, fatty acid/glycerol molar ratio 1.2
VIII	10% methanol in ethyl ether	2500	Unknown	530	5.1	Dark red solid, probably peroxidized squalene and carotene degradation products

* 10.63 g. of lipid was applied to 600 g. of silicic acid. The total recovery was 10.59 g. (100%). See Figure 2 for the elution pattern.

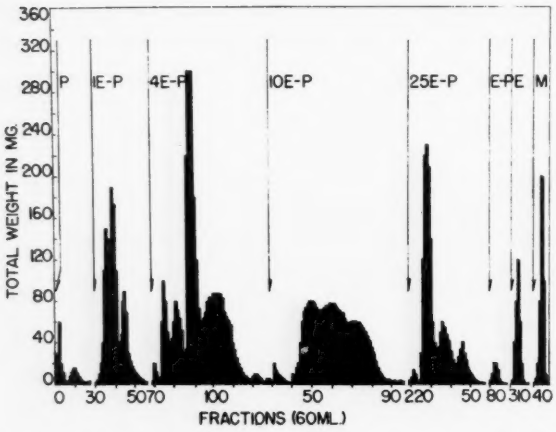

FIG. 2. Chromatography on silicic acid of the neutral lipids (low in free sterols) from corn oil distillate 1 D. Solvents as in Figure 1. E-P indicates 50 per cent diethyl ether in petroleum ether. The composition of the various fractions is recorded in Table 5.

Partition Chromatographic Fractionation of Components Isolated from the Distillates Following Adsorption Chromatography. Because of the nature of the project (see first two paragraphs) of which this study was a part, interest was focused primarily on the free sterols and sterol esters. As a result of certain observations during the preliminary paper chromatography of lipid mixtures rich in sterol esters, it was noticed that the application of reversed phase paper partition chromatography would provide a quick indication of the nature of the sterol esters present. Several solvent systems were developed which permitted a satisfactory resolution of all the even-numbered fatty acid esters of a given plant sterol (12). Difficulties, however, were observed when several esterified sterols were present. Thus, in the case of the corn oil sterol esters, at least three different sterols provided the alcohol parts of the esters. When such an ester mixture was chromatographed in these systems, several discrete bands were obtained (Fig. 3). In order to identify these bands, it was necessary to collect at least sufficient amounts of material for saponification and subsequent paper-chromatographic analyses of the hydrolysis products. This was accomplished by scaling up the above reversed phase paper partition chromatography method for preparative purposes. Using this method, as much as 50 mg. of the mixed sterol esters were eventually separated into the different bands (Fig. 3). The bands were eluted, the lipids saponified, and the fatty acids and the unsaponifiable matter recovered separately. The acids were chromatographed in the reversed phase systems described by Schlenk *et al.* (14) and Inouye and Noda (15). The latter method permitted the resolution of the "inseparable" pairs of the fatty acids and a semiquantitative estimation. The unsaponifiable parts of the esters were chromatographed, using a modification of the method described by Sulser and Hoegl (13). It permitted the separation of beta- and gamma-sitosterol, the alpha-sitosterol(s), saturated sterols, and tocopherols present in the unsaponifiable matter from the mixed sterol esters (Fig. 4).

As a result of these analyses, it was possible to demonstrate that the separation of the esters (Fig. 3) had taken place primarily on the basis of the fatty acid moieties. Thus the beta- and gamma-sitosteryl oleates had traveled together (band I) but had overlapped to some extent with the palmitic acid derivatives. The next fastest moving band, H, was that of beta- and gamma-sitosteryl linoleates. Figure 5 shows the sterol composition of the mixed sitosteryl linoleate fraction from band H. An identical chromatogram was obtained for the sterols present in the mixed oleate fraction (band I). The next band, G, in Figure 3, was due to the oleate of a sterol moving faster than either beta- or gamma-sitosterol, assumed to be alpha-sitosterol, and minor amounts of as yet unidentified fatty acid esters of beta- and gamma-sitosterols. This band also contained some tocopherol ester which, on the basis of its R_f value, was believed to be the oleate. The composition of the sterols of band G is shown in Figure 6. The fastest moving ester band, F, in Figure 3 was thought to be due to the linoleate of the alpha-sitosterol and another tocopherol ester, possibly the linoleate. The fatty acid part of band F also contained at least one additional unsaturated fatty acid other than linolenic, which moved faster than linoleic.

The sterol bands were identified on the basis of their color reactions and the R_f values. The reliability of the latter criterion was checked by performing the chromatographic separations of the unknowns with and without the addition of known standards of the beta- and gamma-sitosterols. In the absence of any alpha-sitosterol standards, the identification of the alpha-sitosterol band is only tentative. Anderson and Shriner (22) suggested that alpha-sitosterol occurs in corn oil in fair amounts and this suggestion has been accepted by us as a working hypothesis. That this band was not due to stigmasterol was demonstrated by the observation that the latter formed a distinct and somewhat faster moving band than the unknown compound. The triterpene dienol structure recently suggested for alpha-sitosterols by Mazur *et al.* (40)

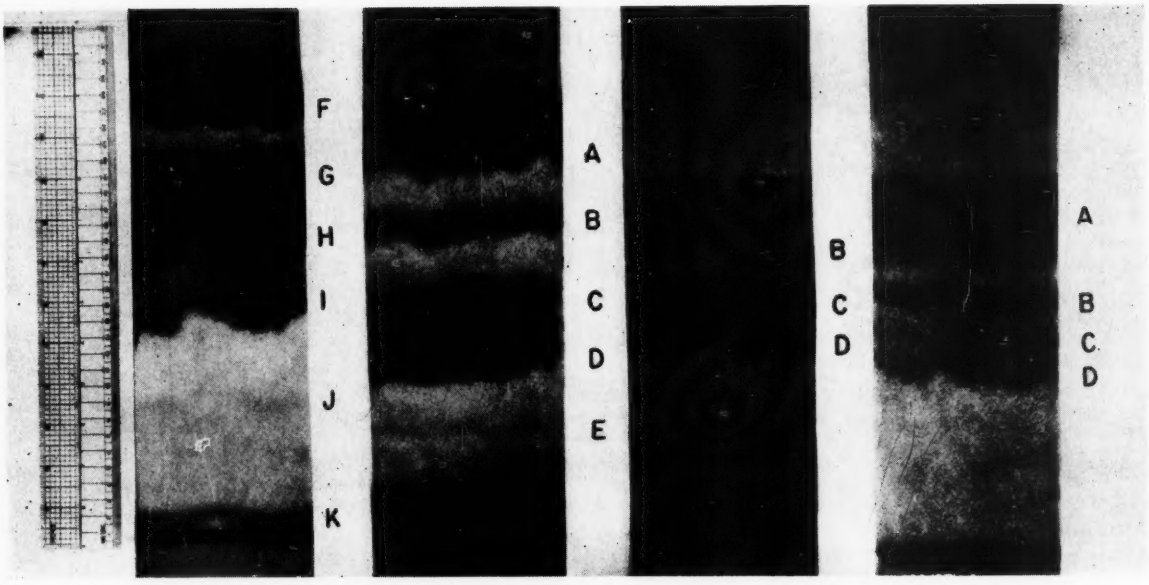

FIG. 3. Chromatography of corn oil tocopherol and sterol esters. System: 90% propionic acid/paraffin oil on paraffinized Whatman No. 1 filter paper. Chromatogram stained with iodine vapor. F = tocopherol and alpha-sitosteryl linoleate (tentative), G = alpha-sitosteryl oleate, H = beta- and gamma-sitosteryl linoleates, I = beta- and gamma-sitosteryl oleates and palmitates, J = unknown, K =unidentified esters of beta- and gamma-sitosterols.

FIG. 4. Chromatography of free tocopherols and sterols from the corn oil sterol ester hydrolysate. System: 88% acetic acid/paraffin oil on paraffinized Whatman No. 1 filter paper. Chromatogram stained with iodine vapor. A = tocopherols, B = alpha-sitosterol(s), C = gamma-sitosterol, D = beta-sitosterol, E = saturated sterol (?).

FIG. 5. Chromatography of free sterols from the pooled steryl linoleate (band H, Fig. 3) hydrolysate. System and code as in Figure 4.

FIG. 6. Chromatography of free sterols from the pooled alpha-sitosteryl oleate (band G, Fig. 3) hydrolysate. System and code as in Figure 4.

might account for the slightly lower polarity of this compound in comparison to stigmasterol in the reversed phase partition system. The tocopherol bands were identified after elution and determination of the ultraviolet spectra. On paper, these bands first turned yellow, then red, after standing in the air for periods of 24 hours or more.

A similar paper chromatographic examination of the sterol present in the free form in the corn oil distillates showed the predominance of the beta- and gamma-sitosterols. The sterols obtained on crystallization of 1 D gave only two readily detectable sterol bands, one for beta- the other for gamma-sitosterol. The free sterols obtained on adsorption chromatography of the neutral lipids from the various distillates on silicic acid contained, in addition to these two sterols, also significant quantities of the alpha-sitosterol. Though the sterol and tocopherol bands obtained on reversed phase paper partition chromatography appeared to be fairly sharp and distinct (Figs. 4, 5, and 6), it was felt that they could have hidden other minor components. It has been the experience of the authors with the reversed phase partition systems (12) that only suitably proportioned solute concentrations may be satisfactorily partitioned. A large excess of one component readily masks the minor component unless they differ greatly in their polarities. Therefore attempts are currently being made to effect an enrichment of the minor sterol and sterol ester components so that more satisfactory chromatographic examination may be performed.

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NH_2-terminal amino acids of the serum lipoproteins of normal and hypercholesterolemic rabbits*

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SUMMARY

Aspartic acid is the major and glutamic acid and serine are minor NH_2-terminal amino acids of the lipoproteins of rabbit serum. No NH_2-terminal amino acids other than these are found after prolonged cholesterol feeding. The hyperlipoproteinemia accompanying the feeding is not characterized by any significant changes in the NH_2-terminal amino acid composition of the density (d) $<$ 1.007, 1.007 to 1.019, or 1.065 to 1.20 lipoproteins. However, in the d = 1.019 to 1.065 lipoproteins, the percentages of NH_2-terminal glutamic acid and aspartic acid seem to increase and decrease, respectively, during feeding.

The atherosclerosis induced experimentally in the rabbit by prolonged cholesterol feeding is accompanied by hypercholesterolemia and hyperlipoproteinemia. Ultracentrifugal studies have demonstrated a pronounced increase in concentration of low density serum lipoproteins during cholesterol feeding (1). Whether this increase reflects an increase in concentration of some or all of the lipoproteins normally present in rabbit serum, or whether it reflects the synthesis of lipoproteins not present previously, cannot be answered from ultracentrifugal studies alone, since lipoproteins of very similar or even identical sedimentation characteristics need not be chemically identical. As an additional means of answering this question, we have chosen to compare the NH_2-terminal amino acids of the serum lipoproteins before and at various intervals during cholesterol feeding. Thus a quantitative difference in NH_2-terminal amino acid content is evidence for a quantitative difference in the lipoproteins. Knowledge of the terminal amino acids should indicate some of the possible metabolic interconversions in an animal which is used extensively in studies of experimental atherosclerosis.

EXPERIMENTAL

New Zealand white female rabbits were fed Purina rabbit pellets containing 1 per cent cholesterol. The cholesterol was dissolved in ether, the solution was added slowly to the pellets with mixing, and the solvent was allowed to evaporate. For isolation of lipoproteins, 10 to 30 ml. blood were drawn from the ear vein.

Ultracentrifugal Isolation and Analysis. Lipoproteins were isolated from rabbit serum by centrifugation in solutions of varying density (2). The d $<$ 1.007 fraction[1] contained lipoproteins of hydrated density less than 1.007 g. per ml., and the d = 1.007 to 1.019, 1.019 to 1.065, and 1.065 to 1.20 g. per ml. fractions contained those of hydrated densities between the relevant solvent density values. The fractions were characterized by the density of the medium used in isolating them and by analysis in the Model E Spinco analytical ultracentrifuge. Weight average flotation coefficients were obtained by dividing the schlieren pattern into narrow strips, determining the concentration (c_i) and flotation coefficient (S_{f_i}) of each strip in the customary manner, and evaluating the quotient $\sum_i S_{f_i} c_i / \sum_i c_i$. The flotation coefficients were not corrected to zero concentration, nor were Johnston-Ogston corrections applied. However, by means of a Brice-Phoenix differential refractometer, and with the assumption that the specific refractive increment of the low density, d $<$ 1.065 lipoproteins was identical with that (0.154 ml. per g.) of the d $<$ 1.065 lipo-

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[1] Abbreviations used: d, density; S_f, flotation coefficient, the negative of the sedimentation coefficient in Svedberg units of species undergoing centripetal migration in a sodium chloride solution of density 1.0630 g./ml. at 26°C; and DNP, dinitrophenyl.

proteins of human serum in a NaCl solution of density 1.0630 g. per ml. at 26°C, the lipoprotein concentration of identical density fractions obtained during the experiment was kept constant for ultracentrifugal analysis. Thus, for purposes of relative comparison, a comparison of successive S_f values should be almost as meaningful as one of S_f^o values. The d < 1.007, 1.007 to 1.019, 1.019 to 1.065, and 1.065 to 1.20 g. per ml. fractions contained the S_f 20 and greater, S_f 12-20, S_f 0-12, and high density lipoproteins of rabbit serum, respectively.

Terminal Amino Acid Analysis. NH_2-terminal amino acid analysis by the dinitrofluorobenzene technique (3) was carried out with the same general procedures used previously with human serum lipoproteins (2). The only differences in technique were the use of a 180-minute reaction period at room temperature for the mixture of 1 volume aqueous lipoprotein solution containing 1 per cent $NaHCO_3$ and 2 volumes of 2.5 per cent 2,4-dinitrofluorobenzene in ethanol, and the use of a 16-hour hydrolysis at 105°C with the redistilled HCl. After reaction with the dinitrofluorobenzene, the DNP-lipoprotein was washed with water and with combinations of ethyl ether, ethanol, methanol, acetone, methylal, and chloroform. Corrections for loss of NH_2-terminal amino acids during hydrolysis and subsequent procedures were made with the assumption that their recoveries from the protein were the same as those for the corresponding free DNP-amino acids under the same experimental conditions. A known amount of the relevant DNP-amino acid[2] was added to a weighed sample of DNP-protein. Hydrolysis, extraction, chromatography, and measurement of the optical density of the chromatogram eluate were carried out simultaneously with a weighed sample of DNP-protein without added DNP-amino acid. Subtraction of the amount of DNP-amino acid derived from the protein gave recoveries of 70 to 73, 76, and 65 to 68 per cent, respectively, for the added DNP-aspartic acid, DNP-glutamic acid, and DNP-serine. Essentially the same recoveries were obtained for these DNP-amino acids analyzed in the absence of protein. Added DNP-glutamic acid did not alter the yield of DNP-aspartic acid from DNP-protein, and added DNP-aspartic acid did not alter the yield of DNP-glutamic acid.

RESULTS AND DISCUSSION

Ultracentrifugal Analysis. The sedimentation coefficients of the lipoproteins during a representative experiment are summarized in Table 1. As observed by others (1), both the S_f rate and concentration of the d < 1.065 lipoproteins increased monotonically with continued feeding. The most pronounced increase in concentration occurred in the lipoproteins with S_f values of 20 or more, with serum levels of 1500 mg. per 100 ml. not uncommon after 12 weeks. After this period of feeding, the S_f 0-12 levels were about 200 mg. per 100 ml. Aortal plaques characteristic of cholesterol-fed rabbits appeared in most of the animals after 4½ weeks of feeding. None of the aortas were spared the plaques after 8 weeks, indicating that experimental atherosclerosis had certainly been achieved by this time. Plaques were still present in the aortas of rabbits fed a normal diet for 24 weeks after 16 weeks of cholesterol feeding. Low density serum lipoprotein levels in these rabbits had returned to values 10 to 20 mg. per 100 ml. above those (< 40 mg. per 100 ml.) before feeding.

Terminal Amino Acid Analysis. A study of terminal amino acid changes in the lipoproteins of a single animal during the feeding period would have been complicated by the increase in concentration of low density lipoproteins that follows extensive blood loss (4). Neither the differences in data obtained on lipoproteins from pools of 3, 5, and 7 rabbit sera nor that between duplicate analyses of the same sample was greater than 10 per cent. With animals fed cholesterol for 9 or for 15 weeks, 80 ml. blood yielded enough material for analysis of the d < 1.007, 1.019 to 1.065, and 1.065 to 1.20 lipoprotein fractions.[3] The differences were less than 10 per cent between these data from a single animal and those on similar lipoproteins from pooled blood of 3 and 7 rabbits fed cholesterol for 9 and 15 weeks. It is thus unlikely that the significance of the values of Table 1 was made uncertain by the necessity of using pooled blood.

The NH_2-terminal amino acid composition of the lipoproteins of rabbit serum before and during cholesterol feeding, and 24 weeks after the end of feeding and restoration to a normal diet, is summarized in Table 1. In addition to the NH_2-terminal amino acids given in Table 1, smaller amounts (1.0 to 2.5 $\times$ 10^6 g. protein per mole serine) of DNP-serine were found in all the lipoprotein fractions. Since these amino acids occur in phospholipids, one must be concerned whether the extensive washings with the previously mentioned combinations of organic solvents were effective in removing phospholipids from the DNP-protein before

[2] Mann Research Biochemicals, New York, N. Y.

[3] These rabbits were removed from the experiment after the bleeding.

TABLE 1. NH_2-TERMINAL AMINO ACIDS OF THE SERUM LIPOPROTEINS OF NORMAL AND HYPERLIPOPROTEINEMIC RABBITS

Density	S_f	Weeks on Diet*	G. Protein/Mole NH_2-Terminal Amino Acid	
			Aspartic Acid	Glutamic Acid
g./ml.				
<1.007	29	0	6.7×10^4	2.7×10^5
	40	4½	8.2×10^4	3.6×10^5
	51	9	7.5×10^4	3.6×10^5
	67	15	8.2×10^4	2.9×10^5
	32	15†	7.1×10^4	2.7×10^5
1.007–1.019	15.0	4½	1.7×10^5	5.6×10^5
	17.2	9	1.7×10^5	4.6×10^5
	18.7	15	1.8×10^5	4.8×10^5
1.019–1.065	6.4	0	2.1×10^5	7.4×10^5
	7.3	4½	2.4×10^5	5.4×10^5
	9.3	9	2.6×10^5	4.2×10^5
	10.2	15	2.6×10^5	3.9×10^5
	6.2	15†	2.2×10^5	7.6×10^5
1.065–1.20		0	4.0×10^4	
		9	3.9×10^4	
		15	4.2×10^4	2.0×10^6

* Rabbit pellets containing 1% cholesterol.

† These lipoproteins were obtained from animals kept on rabbit pellets 24 weeks after having been fed the cholesterol diet 15 weeks.

hydrolysis. The following experiment suggests that phospholipids, except possibly tightly bound ones, were effectively removed.

Male rats were injected with 200 μc. P^{32} orthophosphate. Sixteen hours later their livers were removed, washed with isotonic saline, and extracted with methylal, methanol, chloroform, and ethyl ether. The lipid extract was then fractionated on silicic acid columns (5) and 99.8 per cent of the radioactivity added to the column was found in the methanol eluate. Infrared spectrophotometry (5) confirmed the phospholipid nature of the eluate. An amount of phospholipid corresponding to 25 per cent by weight of the lipoprotein fraction was added to $d < 1.007$ and $d = 1.019$ to 1.065 lipoproteins of known terminal amino acid content, and the mixture was reacted with 2,4-dinitrofluorobenzene. The P^{32} content of the DNP-protein after washing, which was less than 0.08 per cent of the radioactivity associated with the insoluble lipoprotein after reaction with dinitrofluorobenzene, and the subsequent quantitative terminal amino acid determinations indicated that phospholipid contaminant could have accounted for 20 to 40 per cent of the NH_2-terminal serine, but for only a minor (less than 10 per cent) amount of the terminal aspartic or glutamic acids found.

These data support the peptide origin of at least aspartic and glutamic acids. Delipidation of rabbit lipoproteins prior to reaction with dinitrofluorobenzene was not done in this study. However, quantitative NH_2-terminal amino acid determination of S_f 6-8 and high density human serum lipoproteins was not affected by delipidation prior or subsequent to reaction with dinitrofluorobenzene.[4] Nevertheless, it can be argued that until the lipoproteins have been separated into discrete NH_2-terminal aspartic acid, glutamic acid, and serine containing lipoproteins, and NH_2-terminal peptide fragments containing the relevant amino acid have been isolated from the lipoproteins, definite conclusions regarding the peptide origin of all the NH_2-terminal amino acids found and the lengths of the peptide chains from which they came cannot be made. Reasonable estimates of lipoprotein molecular weights and protein content give values for the molecular weights of the protein moieties which are less than the values of g. protein per mole NH_2-terminal serine, except possibly in the $d < 1.007$ and 1.007 to 1.019 lipoproteins. The serine found could possibly represent a lipoprotein or lipoproteins present in relatively low concentration, a contaminating protein, or phospholipid.

The presence of a common protein component in the rabbit lipoproteins, as suggested by the common NH_2-aspartic acid, would be compatible with metabolic interconversions not of a proteolytic nature. Thus one lipoprotein could be converted to another by processes which remove lipids, such as the action of lipases or phospholipases or the transfer of lipids to other protein or lipoprotein acceptors. As may be seen from Table 1, considerable variation in the g. protein per mole NH_2-aspartic acid exists among the various lipoproteins. The variation in the amount of NH_2-aspartic acid in the protein components of the low density lipoproteins might be attributable to the presence in varying amounts of an NH_2-glutamic acid chain. However, since the $d < 1.007$ protein has greater percentages of both NH_2-terminal aspartic and glutamic acids than are found in the 1.007 to 1.019 or 1.019 to 1.065 proteins, this seems unlikely. Our data thus seem to indicate that two or more peptide chains of different molecular weight and the same end group are present

[4] B. Shore and V. Shore, unpublished data.

in these lipoproteins. In short, the data, while not eliminating the presence of a common protein chain or chains in the lipoproteins, do not give positive support for their existence.

The occurrence after cholesterol feeding of an NH_2-terminal amino acid not present before feeding would of course have been good evidence for the synthesis of a new lipoprotein. As may be seen from Table 1, aspartic acid, glutamic acid, and serine were the only NH_2-terminal amino acids found throughout the entire experimental period.

The data of Table 1 indicate that the quantitative NH_2-terminal aspartic acid content of the d = 1.065 to 1.20 high density lipoproteins of rabbit serum does not change throughout the cholesterol feeding. If we assume that only one protein chain is present, then the minimum molecular weights of the NH_2-terminal aspartic acid containing proteins of the d = 1.065 to 1.20 lipoproteins of three species are not greatly different: 40,000[5] or 54,000 (6) in man, 46,000 (7) in the dog, and 40,000 in the rabbit. Table 2 presents the comparative biochemistry of the NH_2-terminal amino acids of the serum lipoproteins of various species. In contrast to the other species, aspartic acid is the major NH_2-terminal amino acid of all of the lipoproteins of rabbit serum.

TABLE 2. NH_2-TERMINAL AMINO ACIDS OF SERUM LIPOPROTEINS OF VARIOUS SPECIES

Density of Lipoprotein	Species*	NH_2-Terminal Amino Acids	
		Principal Components	Minor Components†
g./ml.			
<1.007	Man (2, 6, 9)	Serine, threonine, aspartic acid	Glutamic acid
	Rabbit	Aspartic acid	Glutamic acid, serine
1.007–1.019	Man (6)	Serine, threonine, aspartic acid, glutamic acid‡	
	Rabbit	Aspartic acid, glutamic acid	Serine
1.019–1.065	Man (2, 6, 8, 9)	Glutamic acid	Serine, threonine, aspartic acid
	Rabbit	Aspartic acid	Glutamic acid
	Dog (7)	Glutamic acid	
1.065–1.20	Man (2, 6, 8, 9, 10)	Aspartic acid	Serine, threonine
	Rabbit	Aspartic acid	
	Dog (7)	Aspartic acid	

* Numbers in parentheses refer to References.

† Refers to NH_2-terminal amino acids which were less than 20 per cent of the total moles of amino acid recovered from the protein analyzed.

‡ Data from Reference 6 and our unpublished data.

Because the terminal amino acid composition of the d < 1.007 lipoproteins fluctuated irregularly rather than monotonically, it is difficult and perhaps unwise to attempt to attach any significance to these data. Any variation in NH_2-terminal groups may be merely a reflection of a variation in the relative amounts of the lipoproteins comprising this spectrum of molecules. Indeed, the ultracentrifugal data indicate a shift to higher S_f values and a tremendous increase in concentration in this heterogeneous fraction following cholesterol feeding. Insufficient d = 1.007 to 1.019 lipoproteins from animals fed no cholesterol was available for comparison, but there were no quantitative differences in terminal amino acids among samples 4½, 9, and 15 weeks after feeding.

During cholesterol feeding there were consistently monotonic increases in NH_2-glutamic acid composition and decreases in NH_2-aspartic acid composition of the d = 1.019 to 1.065 lipoproteins. As Table 1 indicates, after the rabbits were returned to a normal diet for 24 weeks after the 15 weeks on cholesterol, the relative proportion in their 1.019 to 1.065 lipoproteins of the NH_2-terminal aspartic and glutamic acid lipoproteins, which had been changing throughout the feeding period, became that before feeding.

It is clear that comparison of ultracentrifugal patterns and NH_2-terminal amino acid content is a first step in distinguishing among closely related lipoproteins. It seems necessary to supplement these data with immunochemical studies, with proteolysis and two-dimensional chromatography-electrophoresis of the resultant peptides (7, 11), and with other chemical and physical procedures. These techniques should also be valuable in deciding whether any lipoprotein fractions of differing density have a common NH_2-terminal aspartic acid protein chain.

[5] Better recoveries of the NH_2-terminal amino acids in our more recent studies indicate that an average chain length of 40,000 is a more reliable value than that published previously (2).

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Uptake and metabolism of triglycerides by the rat liver*

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SUMMARY

Triglycerides were prepared with C^{14} in the glycerol moiety and tritium in the fatty acid part as well as with over-all tritium labeling. Tracer amounts of these triglycerides were incorporated into blood plasma lipoproteins and the solutions were injected intravenously into rats. Large amounts of the radioactive material were recovered in the liver 15 minutes after the injection and the triglycerides were located predominantly in the liver mitochondria and microsomes, with relatively little in the fat droplets. Five minutes after the injection of doubly labeled triglycerides, the triglycerides recovered in the liver had practically the same H^3- to C^{14}-ratio as the injected material, indicating that intravenous lipolysis is not necessary for the uptake of neutral fat by the liver. After longer intervals the H^3- to C^{14}-ratio in the triglycerides increased rapidly, indicating intrahepatic lipolysis with the loss of free glycerol and re-esterification of the fatty acid with endogenous unlabeled glycerol precursors. Part of the tritiated fatty acids was also recovered in the phospholipid fraction, while very little of the C^{14}-glycerol was used in phospholipid synthesis.

In a previous publication (1) it was shown that free fatty acids (FFR), injected intravenously in tracer amounts, are rapidly removed by the liver and can be recovered to a large extent as triglycerides, located at the sites of their synthesis, i.e., the microsomes and the mitochondria (2). It was shown by perfusion experiments (1) that the triglycerides are gradually removed from these particles and transferred to the "fat droplet fraction" of the liver or released into the blood. After some time, part of the triglyceride fatty acids was also found in the liver phospholipids. Similar results were also reported by Laurell (3), who showed that blood plasma triglycerides found after injection of radioactive palmitic acid are derived from liver triglycerides.

To complete our knowledge of the transport of the fatty acids, it was therefore of importance to follow the fate of plasma triglycerides. These compounds are introduced into the blood from the lymphatic ducts with the chylomicrons and from the liver as lipoproteins. Other sources are possible but have not yet been satisfactorily demonstrated.

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The metabolism of chylomicrons obtained from the lymph of animals fed with labeled fatty acid has been studied in several laboratories (4, 5, 6). The recent literature on this topic, as well as on work in which artificial fat emulsions were used, has been reviewed by Fredrickson and Gordon (7).

Much less information is available on the fate of the endogenous triglycerides which circulate in the blood as a constituent of lipoproteins. They have been labeled by the injection of precursors, like acetic acid or higher fatty acid (3, 8 to 11) and their turnover has been measured and found to exceed that of the plasma phospholipids.

In the present work labeled triglycerides incorporated into plasma lipoproteins were injected intravenously and their disposition was studied. It soon became apparent that with this material, as was previously found with FFA (1) or with lymph chylomicrons (6), a high percentage of the radioactivity was recovered in the liver. The paper presented here therefore deals solely with the uptake and metabolism of the triglycerides by the liver.

The following points have been investigated: What cytoplasmic particles are the acceptors of plasma triglycerides? Is cleavage to FFA a necessary pre-

liminary reaction to their penetration into the tissue? Does cleavage occur in the organ after penetration of the triglycerides?

In order to test these points it was necessary to inject into the blood stream triglycerides in a form representative of their natural state in the plasma, i.e., as lipoproteins or chylomicrons. By the use of tritiated fatty acids of high specific activity, it was possible to incorporate very small amounts of triglycerides into the plasma lipids without any marked change in their quantity or quality. By high speed centrifugation, the labeled plasma lipids could also be separated arbitrarily into fractions with finer and coarser particles; and possible differences in behavior, due to the physical state of the compounds, could be tested. Finally, since one of the aims of this work was to determine the extent of intravascular lipolysis ("clearing") accompanying the uptake of the triglycerides, these substances were synthesized from C^{14}-glycerol and H^3-fatty acid. With these doubly labeled compounds a change in the H^3- to C^{14}-ratio would be expected in the case of lipolysis. Partial lipolysis and replacement of the H^3-fatty acids with unlabeled endogenous ones would bring about a drop in this ratio. Complete cleavage, on the other hand, would cause a rise in this ratio, as free glycerol is converted to a large extent into carbohydrate metabolites and the fatty acids would be re-esterified with endogenous glycerol precursors.

MATERIAL AND METHODS

Preparation of Labeled Triglycerides. Three types of labeled triglycerides were prepared and used in this investigation with similar results. Tritiated triolein was prepared by tritiating 30 to 40 mg. triolein according to Wilzbach (12). In view of the findings of Nystrom *et al.* (13), it may be assumed that tritiation resulted in partial saturation of the double bond and that a mixture of oleate and stearate was obtained. The triolein, spread in a thin layer, was exposed to 10 c. of tritium gas for 10 days. After removing the bulk of the gas, the material was dissolved in ethanol, allowed to stand for several hours to equilibrate the exchangeable tritium, and the ethanol was then evaporated. Solution in ethanol and evaporation were repeated several times. The product was then taken up in ether and the solution washed twice with a 1 per cent aqueous solution of sodium carbonate to remove water-soluble breakdown products and fatty acids. The residue in the ether solution was chromatographed on a silicic acid column according to Borgström (14), and the triglyceride fraction eluted by benzene was used for the experiments reported.

Doubly labeled triglyceride (glyceryl-1-C^{14} triolate-H^3) was prepared by esterifying glycerol-1-C^{14} with the H^3-fatty acid obtained by saponification of the tritiated triolein. Esterification was carried out by interacting the tritiated fatty acid with glycerol-1-C^{14}.[1] To 30 μmoles glycerol with 100 μc. C^{14} the tritiated fatty acid with carrier was added to make a three- to fourfold excess over the theoretically required amount. The mixture was heated in a closed vessel to 180°C for 12 hours in the presence of small amounts of $SnCl_2$ as catalyst and concentrated sulfuric acid in a side arm to remove the water produced. This was preferred to flushing through with dry CO_2, generally recommended for this esterification, since it was found that with the small amounts of glycerol used, the loss of glycerol by evaporation was considerable. The reaction mixture was extracted with petroleum ether and the extract was washed repeatedly with water. The petroleum ether was evaporated and the residue was dissolved in anhydrous acetone and passed through a column of MgO and Celite (1:1) to remove the unesterified oleic acid. Several μmoles of fatty acid carrier were added and the acetone solution was passed again through a MgO-Celite column. The acetone eluate was evaporated and the residue dissolved in petroleum ether. This solution was then chromatographed on a silicic acid column according to Borgström (14) to separate the triglycerides from the lower glycerides and other contaminants.

Glycerol-1-C^{14} tripalmitate-9,10-H^3 was prepared as described for the previous preparation, using palmitic acid-9,10-H^3.[2]

Labeled Lipoproteins. Incorporation of the glycerides into plasma lipoproteins was achieved by adding 0.05 ml. of an alcoholic solution of the labeled triglyceride (containing not more than 0.2 μmoles) to 13 ml. fresh rat serum and incubating for 30 to 60 minutes at 37°C with shaking. The lipoproteins were then fractionated by chilling the serum in ice and centrifuging at 100,000 $\times$ g for 60 minutes. The bottom of the plastic centrifuge tube was punctured and the lowest 3 ml. collected (fraction 1). Two more fractions of 3 ml. were obtained from the middle of the tube (fractions 2 and 3) and finally the upper layer (fraction 4) was collected. The last fraction was usually turbid and contained the serum chylomicrons.

[1] Obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

[2] See footnote 1.

For technical reasons the method of centrifugal fractionation used was an arbitrary one, and the fractions obtained did not coincide with the generally accepted division of lipoprotein fractions. The aim of this fractionation was solely to establish whether any gross difference appears with lipoproteins of different degrees of dispersion.

Paper electrophoresis of these serum fractions showed that the bulk of the radioactivity migrated with the β lipoprotein and the rest stuck to the point of origin. No detectable quantities migrated with the serum albumin, indicating that no significant lipolysis occurred during the incubation of triglycerides in the sera.

The experimental procedure consisted of injecting 0.5 to 1.0 ml. of the serum fractions into the tail vein of male rats weighing 100 to 120 g., fed on an ordinary stock diet. At the indicated time intervals the animals were bled under light ether anesthesia from the abdominal aorta. The liver was chilled immediately in crushed ice and homogenized in KCl-Tris buffer and fractionated by centrifugation into debris (comprising intact cells, nuclei, and trapped cytoplasmic particles), mitochondria, supernatant (containing the microsomes), and floating fat, as described in a previous publication (2). Extraction and fractionation of the lipids were also performed as described in that paper (2).

Assay of radioactivity was performed with a Packard Tri-Carb liquid scintillation counter in which simultaneous counts of H^3 and C^{14} were determined. The degree of quenching was examined in the triglyceride and in phospholipid fractions by introducing internal standards. With the former this was negligible, and with the latter it was minimized by using low concentrations.

RESULTS

When emulsions of triglycerides were injected intravenously, a considerable portion of the injected material was found in the liver 15 minutes after the injection. This was the case when the highly dispersed lipoprotein fraction (fraction 1) was used, as well as when the coarser serum chylomicron fraction (fraction 4) was injected (Table 1). Similar results were obtained by Bragdon and Gordon (6) when lymph chylomicrons were injected. It has been observed that this deposition in the liver is the rule whenever well-dispersed fatty compounds are injected. When coarser, artificial fat emulsions are used, considerable portions can be found in the lungs. This has been found to be an indicator for the adequacy of emulsification in intravenous lipid applications. Furthermore, the results presented show that the triglycerides penetrating the liver cell are deposited primarily in the metabolically active cytoplasmic particles of the liver, as was previously shown for the glycerides formed from injected FFA (1). The fat droplets of the liver contained relatively little radioactivity when examined 15 minutes after the injection. As with the triglycerides derived from FFA, here too the distribution of the triglycerides is gradually shifted; and larger amounts can be found in the free fat as time progresses. There was no gross difference in the behavior of the various serum fractions. Fraction 4, which contained most of the serum chylomicrons, was taken up by the liver at a more rapid rate and larger portions were found in the droplets with these lipid emulsions.

TABLE 1. DISTRIBUTION OF TRITIATED TRIOLEIN IN RAT LIVER 15 MINUTES AFTER INJECTION

Serum Fraction Injected*	Per Cent of Injected Dose			
	Whole Liver	Mitochondria	Supernatant†	Fat‡
1	24	5	7.5	0.4
2	27	5	10	0.3
3	26	7	10	0.3
4	60	10	15	4.0

* For preparation of fractions see Materials and Methods in text.

† Supernatant at 20,000 $\times$ g for 30 minutes.

‡ Combined fatty layers separated after each centrifugation.

The figures given in Table 1 for distribution in the various particles are not absolute and indicate only the relative distribution in the various fractions. They do not add up to the total amount found in the liver, since considerable amounts are present in the debris, obtained after low speed centrifugation, i.e., in the fraction containing intact cells, cell nuclei, and also trapped cytoplasmic particles. It should further be remarked that the glycerides found in the supernatant, obtained after centrifugation at 20,000 $\times$ g, are mainly bound to the microsome fraction and can be precipitated by prolonged centrifugation in the Spinco centrifuge at 100,000 $\times$ g. This was done only in a

few experiments and the results are presented as "supernatant."

In Table 2 the results of experiments are given in which doubly labeled triglycerides were injected intravenously. There was no significant difference between triglycerides labeled with palmitic acid-9,10-H^3 or with "tritiated oleic acid."

It is evident that the triglycerides, isolated from the liver 5 minutes after the injection, have H^3- to C^{14}-ratios, which are practically identical with those of the substance injected. This ratio increases rapidly and 15 minutes after the injection is already significantly higher than the original one. These results indicate that there was no marked intravascular lipolysis and that the triglycerides were deposited in the liver intact. In this organ, however, a rapid dissociation between the glycerol moiety and the fatty acids takes place, as indicated by the rapid rise in the H^3- to C^{14}-ratio in the glycerides. With this technique no accumulation of lower glycerides was observed, i.e., no temporary drop in the ratio was found.

Concomitant with the increase in the isotope ratio and the decrease in total triglyceride activity there is a gradual rise in the radioactivity of the phospholipid fraction (Table 3). The phospholipids formed from the injected triglycerides have a H^3- to C^{14}-ratio at least five times that of the original triglyceride. This finding indicates that the phospholipids are formed predominantly from the fatty acids derived from triglycerides, and only very little from the glycerol.

TABLE 2. H^3/C^{14} IN RAT LIVER NEUTRAL GLYCERIDES FOLLOWING INJECTION OF LABELED SERUM*

Minutes After Injection	Glyceryl-1-C^{14} tripalmitate-9,10-H^3	Glyceryl-1-C^{14} trioleate-H^3
5	1.18 ± 0.02†	1.05 ± 0.02
15		1.25 ± 0.04
30	1.43 ± 0.12	1.56 ± 0.25
60	1.92 ± 0.08	2.07 ± 0.06
120		2.58 ± 0.08
720		6.0

* Relative to the ratio of the injected material, which is taken as 1.0.

† Standard deviation of the mean, obtained in each case from 3 to 4 experiments, except for the figure for 720 minutes, which is single.

DISCUSSION

The results presented show that triglycerides injected intravenously in the form of serum chylomicrons, or incorporated into lipoproteins, are rapidly removed and that a considerable portion is found in the liver. The localization of these triglycerides in the liver was found to be similar to that found after injection of FFA (1) and indicates that the liver contains at least two compartments for triglycerides: one with a rapid turnover and metabolism situated on the active particles, and a second, slower one in the fat droplets. This compartmentalization may explain the finding of Waddell *et al.* (15) with artificial fat emulsions, and of French and Morris (5) with chylomicron fatty acids, that the clearance of plasma triglyceride decreases rapidly with increasing doses. It is likely that with small doses all the injected material is cleared by the particles. However, when these sites become saturated by increasing loads, the clearance becomes dependent upon the removal of the glycerides from the particles, which is a much slower process.

The experiments with the doubly labeled triglycerides showed that for the penetration of triglycerides into the liver cells, intravascular lipolysis is not re-

TABLE 3. LIVER PHOSPHOLIPIDS AND NEUTRAL GLYCERIDES FOLLOWING INJECTION OF MIXED LABELED TRIGLYCERIDES

Minutes After Injection	Per Cent of Injected Fatty Acids		Per Cent of Injected Glycerol	
	Phospholipids	Glycerides	Phospholipids	Glycerides
5	3.0 ± 0.6*	37 ± 5.8	1.85 ± 0.5	35 ± 6
15		44 ± 4.2		35.5 ± 3.4
30	16 ± 2.8	21 ± 3.6	2.75 ± 0.7	13.5 ± 2.5
60	23 ± 3.6	23 ± 2.4	3.85 ± 0.5	11 ± 1.4
120	15.5 ± 2.4	16 ± 0.8	1.45 ± 0.3	6.2 ± 0.3
720	10	5.5	0.80	0.80

* Standard deviation of the mean, obtained in each case from 3 to 4 experiments, except for the longest period (720 minutes), where only single values are available.

quired since the ratio between labeled glycerol-C^{14} to fatty acid-H^3 in the liver glycerides, shortly after the injection, was the same as that in the injected triglyceride.

It may be claimed that the lipoproteins employed in this study have been altered by the incubation process. The procedure used was, however, extremely mild. After incubation of serum with minute amounts of triglycerides it was demonstrated by paper electrophoresis and high speed centrifugation that the triglyceride was strongly attached to the proteins. Lacking any absolute criteria for establishing the nativity of the lipoproteins, one still has to keep in mind that the material obtained in this way may differ somewhat from the natural one. This reservation does not invalidate our main conclusion, that well-dispersed triglycerides penetrate the liver cells without prior cleavage. Bragdon and Gordon (6) arrived at a similar conclusion on the basis of differences in the tissue distribution of chylomicron triglycerides and FFA. The capability of the liver to ingest intact triglycerides is in agreement with the findings of Courtice and Morris (16), that chylomicrons are transferred freely from the plasma to hepatic lymph.

The present demonstration of a rapid uptake of serum triglycerides by the liver, together with the previous results (1) which showed release of liver triglycerides into the blood, point to a circular process involving the liver and blood serum. Liver and serum triglycerides may therefore be looked upon as constituents of a common pool which equilibrates rapidly. The results of Laurell (3) lend themselves to similar conclusions.

It is known that material is constantly being added to this triglyceride pool by the mobilization of FFA from adipose tissue and their esterification to triglycerides in the liver (1, 3). Several processes are known for the disposal of this pool: Combustion of triglyceride fatty acid to CO_2 has been demonstrated by Bragdon (17) and Morris (18); assimilation of triglycerides by adipose tissue *in vitro* has been demonstrated in our laboratory (19); and Bragdon and Gordon (6) have presented evidence that chylomicron triglycerides, especially after carbohydrate feeding, are taken up by the depots without intravascular hydrolysis. Triglyceride fatty acids are transferred to phospholipids as shown in the present paper, in agreement with Harper *et al.* (9) and with our previous report (1). The comparatively high degree of labeling of the phospholipids makes it unlikely that the triglycerides have to be split into FFA for subsequent reincorporation into phospholipids. In such a pathway the fatty acids would have been diluted to very low activity by the plasma FFA, which were previously shown to be practically devoid of radioactivity, and to mix very rapidly with the liver particles' fatty acid pool (1). The results seem to indicate some mechanism of transesterification, not involving FFA formation.

The glycerol moiety of the liver triglycerides undergoes a much more rapid turnover than the fatty acid part, as is indicated by the rapid increase in the H^3- to C^{14}-ratio in the glyceride fraction. This increase indicates complete cleavage of the triglycerides into free glycerol and re-esterification of the fatty acid moiety with endogenous glycerol precursors. The radioactive glycerol is probably incorporated into the metabolism of carbohydrates. If di- and monoglycerides had accumulated during triglyceride cleavage, a decrease in the H^3- to C^{14}-ratio would have resulted. The fact that at no time could such a drop be detected indicates that the triglycerides which undergo degradation are cleaved predominantly by three successive stages without accumulation of intermediates. This conclusion is corroborated by the finding that the incorporation of the glycerol moiety of the triglycerides into phospholipids is much lower than that of the fatty acid moiety, whereas it would have been expected, according to the scheme of phospholipid synthesis of Kennedy (20), that if diglycerides were formed, they would serve as efficient precursors of phospholipids.

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The effect of changes in nutritional state on the lipolytic activity of rat adipose tissue

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SUMMARY

When intact epididymal fat bodies, obtained from rats given excess carbohydrate, are incubated *in vitro* in the presence of albumin and chyle, free fatty acids (FFA) appear in the incubation medium. These FFA are produced following hydrolysis of the chyle triglyceride by clearing factor lipase present in the fat tissue. When fat tissue obtained from fasted rats is incubated under the same conditions, the amount of FFA appearing in the incubation medium is much less, and it is derived from the tissue rather than from the chyle triglyceride. The function of the clearing factor lipase in adipose tissue is discussed in relation to these findings, and some of the limitations of the study are defined.

It has been reported recently that when intact epididymal fat bodies are incubated with triglyceride in an albumin solution *in vitro*, free fatty acids (FFA) appear in the incubation medium. The production of FFA is highest when the fat tissue is obtained from animals that have been fed carbohydrate, and appears to be due to the action of the clearing factor lipase present in the fat tissue (1, 2). The results of a similar study, in progress in this laboratory, confirm and extend the above findings.

METHODS

Wistar strain male albino rats, weighing between 150 and 200 g., were used. Two groups of rats were studied. The rats in one group were usually fasted for 18 hours, although in certain experiments the period of fasting was increased to 48 hours. Those in the other group were fed their normal diet and were given, at intervals of 1 hour, 3 injections (2 ml. per injection) of glucose (25 per cent) in 0.16 M sodium chloride solution. The injections of glucose were given via the tail vein, with the animals restrained under light ether anesthesia.

After fasting in the one group, or 1 hour after the final injection of glucose in the other, the rats were anesthetized with ether, and from each rat the two epididymal fat bodies were removed. Each body was rinsed in 0.16 M sodium chloride solution, dried lightly on filter paper, and placed in a 25-ml. Erlenmeyer flask containing the incubation medium. The flasks were incubated at 37°C without agitation. The tissue spread out as a thin sheet and presented a large surface area to the medium. Preliminary experiments showed that shaking the flasks did not affect the rate of appearance of FFA in the incubation medium.

In most experiments the incubation medium contained albumin and chyle. Usually 6 ml. of 6 per cent bovine albumin (Armour Fraction V) in 0.16 M sodium chloride solution was used, but in certain experiments the volume, concentration, and pH of the albumin solution were varied. These variations are indicated in the descriptions of individual experiments. The chyle was collected from olive oil-fed rats by the thoracic duct cannulation technique described by Bollman *et al.* (3). The quantity of total esterified fatty acids in the chyle was determined by the method of Stern and Shapiro (4), and the amount of chyle added to the incubation medium was adjusted to give a final concentration of esterified fatty acids in the medium of approximately 6 μmoles per ml. The volume of chyle required was 3 to 5 per cent of the volume of albumin solution.

In experiments in which the nature of the enzyme responsible for the FFA release was investigated, other substances were added to the incubation medium. These included heparin (Pularin-Evans, 100 units per mg.), protamine sulfate,[1] and tetrasodium pyrophosphate.

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[1] Light and Co., Ltd., Colnbrook, Slough.

In most experiments the pH of the incubation medium was adjusted to 8.1 with sodium hydroxide before introducing the fat tissue. Some experiments were carried out at pH 6.0. The albumin in the medium had considerable buffering capacity, and reduction in pH during incubation was small (0.1 to 0.4 units).

When the effect of different agents and conditions on the production of FFA was investigated, one epididymal fat body from each rat was incubated in the test medium and the contralateral body was used as a control. This procedure was necessary since the FFA produced during incubation varied considerably from animal to animal within a group, even though all the animals in the group had received the same preliminary treatment (Table 1). On the other hand, when the two epididymal fat bodies from the same animal were incubated separately under similar conditions, the increases in the FFA content of the medium were in good agreement (Table 1).

In certain experiments the production of FFA was followed when chyle triglyceride was hydrolyzed by the clearing factor lipase of plasma. Plasma containing the clearing factor lipase was obtained from rats injected with heparin (5). The rats were bled 3 minutes after the intravenous injection of 10 units of heparin, and 0.25 ml. of the plasma was added to the incubation medium.

After the fat tissue had been introduced into the incubation medium, the incubation flask was agitated gently for 30 seconds and then a 0.5 to 1.0 ml. sample of the medium was removed. Usually the next sample was taken after 180 minutes' incubation; but in some experiments samples were also taken at intermediate times; and, in one experiment (to show that lipase appeared in the medium during incubation), the tissue was removed after a period and samples of the medium were taken then and also after further incubation.

The FFA were determined in the samples by the method of Dole (6) but phenolphthalein was used as the indicator. To expel all the carbon dioxide from the sample, nitrogen was bubbled through the titration vessel for 30 seconds before beginning the titration. Preliminary experiments showed that observer bias could affect the titration values. Consequently the titrations were carried out in such a manner that the observer was unaware of the nature of the sample being assayed.

Preliminary experiments also showed that under the present experimental conditions the production of FFA was linear over the incubation period but that it was not related in any simple way to the weight of tissue used (Table 1). This last observation was unexpected since in some other studies (1, 7) the production of FFA by intact adipose tissue *in vitro* has been expressed in terms of the wet weight of the tissue. In this study the production of FFA has been expressed as μmoles FFA per hour per epididymal fat body.

In certain experiments the change in the esterified fatty acid content of the medium was determined using the method of Stern and Shapiro (4). Samples of the medium were removed immediately after introducing the fat tissue and 180 minutes later.

RESULTS

The Effects of Fasting and of Glucose Injection on Free Fatty Acid Production. The production of FFA

TABLE 1. PRODUCTION OF FFA WHEN EPIDIDYMAL FAT BODIES ARE INCUBATED *in Vitro* IN THE PRESENCE OF CHYLE AND ALBUMIN

Fasted		Injected with Glucose	
Weight of Tissue	FFA*	Weight of Tissue	FFA*
g.		*g.*	
0.49	0.48	0.52	1.92
0.85	0.40	0.56	2.08
0.42	0.56	0.78	2.00
0.94	0.70	1.25	1.35
0.36	0.59	1.02	2.70
1.13	0.62	0.81	2.35
0.66	0.19	0.50	2.38
0.95	0.38	0.87	4.40
0.64	0.12	0.81	3.92
0.75	0.54	1.05	0.67
0.58	0.49	0.90	1.32
1.10	0.48	0.86	2.30
Mean ± standard deviation	0.46 ± 0.16		2.28 ± 1.1
Left Body*	Right Body*	Left Body*	Right Body*
0.50	0.53	2.56	2.67
0.59	0.61	3.89	3.52
0.40	0.36	2.25	2.70
0.45	0.46	3.56	3.50

* μmoles/fat body/hr.
Upper table: One fat body was used from each animal.
Lower table: Both fat bodies were used.

when intact fat bodies were incubated at 37°C and pH 8.1 in an albumin solution containing chyle is shown in Table 1. The mean rate of appearance of FFA was 0.46 ± 0.16 μmoles per fat body per hour with tissue from fasted rats, and 2.28 ± 1.1 μmoles per fat body per hour with tissue from rats injected with glucose.

The effect on the production of FFA when chyle was omitted from the incubation medium is shown in Table 2. One fat body from each animal was incubated with chyle and the contralateral body was incubated without chyle. In the absence of chyle the production of FFA was markedly reduced when the fat tissue was obtained from animals injected with glucose. On the other hand, with fat tissue from fasted animals, the production of FFA was unchanged. Thus it appeared that in fasted animals the FFA were derived from the fat tissue, whereas in glucose-injected animals the FFA were derived from the chyle triglycerides in the medium.

This was confirmed by determining the change in the esterified fatty acid content of the medium when fat tissue was incubated in the presence of chyle. When tissue from rats injected with glucose was used, the increase in FFA was accompanied by a fall in esterified fatty acid content of the medium, but with tissue from fasted rats, the esterified fatty acid content did not change during the incubation period (Fig. 1).

Also shown in Figure 1 are the changes in the concentrations of free and esterified fatty acids in the medium when clearing factor lipase, in the form of plasma obtained from rats injected with heparin, was incubated with chyle in albumin solution *in vitro*. As expected, the increase in FFA concentration was accompanied by a fall in the esterified fatty acid concentration.

TABLE 2. EFFECT OF CHYLE ON THE PRODUCTION OF FFA* WHEN EPIDIDYMAL FAT BODIES ARE INCUBATED *in Vitro*

Fasted		Injected with Glucose	
With Chyle	Without Chyle	With Chyle	Without Chyle
0.47	0.53	1.32	0.35
0.57	0.56	2.00	0.10
0.70	0.95	1.46	0.20
0.50	0.49	1.36	0.18

* μmoles/fat body/hr.

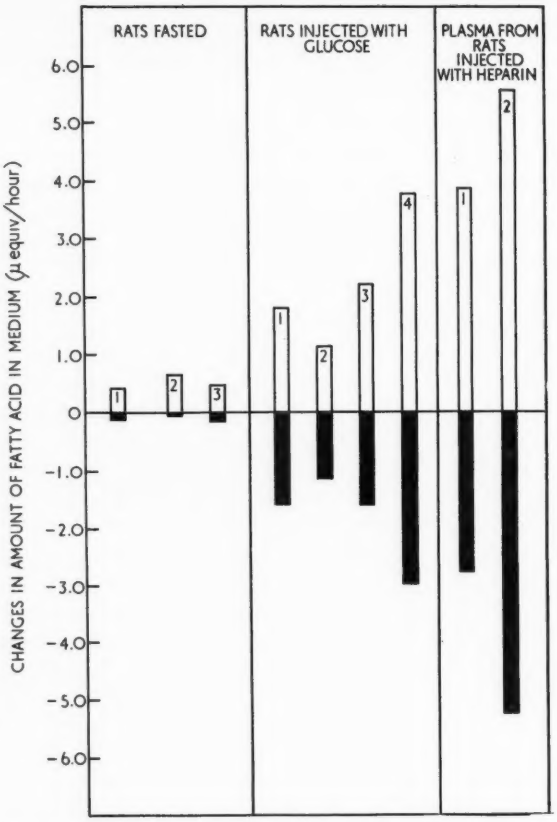

FIG. 1. Changes in the esterified (▪) and FFA (□) content of the incubation medium when (*a*) epididymal fat bodies from fasted rats, (*b*) epididymal fat bodies from rats fed and injected with glucose, and (*c*) plasma from rats injected with heparin were incubated with chyle and albumin *in vitro*. Conditions of incubation as described in the Methods.

The Effect of pH on Free Fatty Acid Production. The results set out in Figure 2 show that when fat bodies from rats injected with glucose were incubated with chyle and albumin, the production of FFA at pH 6.0 was much lower than at pH 8.1. When fat bodies from fasted rats were used, the production of FFA was only slightly reduced at pH 6.0.

The Effect of Inhibitors of the Clearing Factor Lipase on Free Fatty Acid Production. In the presence of protamine sulfate (5 mg. per ml.), sodium pyrophosphate (0.01 M), and sodium chloride solutions (M), the clearing factor lipase in either heparinized plasma or in extracts of adipose tissue is inhibited (8 to 11). These substances also considerably reduce the production of FFA when epididymal fat tissue from rats that have been injected with glucose is incubated in the presence of chyle and albumin (Fig. 3).

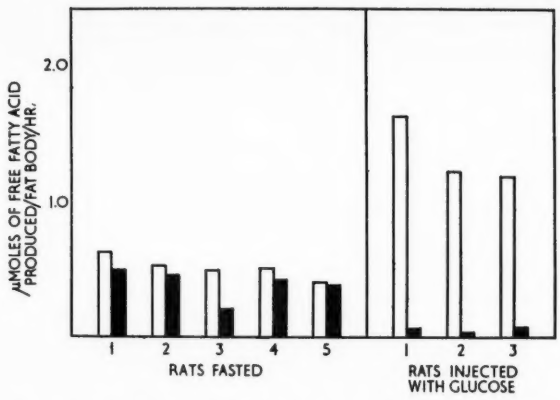

FIG. 2. The production of FFA in the medium when epididymal fat bodies from rats either (*a*) fasted or (*b*) fed and injected with glucose were incubated with chyle and albumin *in vitro* at pH 8.1 (□) and at pH 6.0 (■). Conditions of incubation as described in the Methods.

Their effect on the production of FFA when epididymal fat tissue from fasted rats was incubated under the same conditions was much less.

The Effect of Heparin on Free Fatty Acid Production. Clearing factor lipase appears in the blood following the intravenous injection of heparin (12), and heparin increases the clearing factor lipase activity in aqueous extracts of rat adipose tissue (13). Heparin appears to be an essential component of the complete enzyme system (14) and may be responsible for its stability (15).

The effect of heparin on the production of FFA when epididymal fat tissue from rats, either fasted or injected with glucose, was incubated in the presence of chyle and albumin is shown in Figure 4. In both cases the production of FFA was increased in the presence of heparin. However, the percentage increase was less with fat tissue from fasted fats; and, after 48 hours of fasting, the percentage increase was less than after 18 hours of fasting.

The Effect of Removal of the Fat Tissue from the Medium on Free Fatty Acid Production. Experiments were carried out to determine whether after a preliminary incubation of the fat tissue, FFA continued to be formed in the incubation medium when the fat tissue was removed. The results are shown in Table 3.

When the fat tissue was obtained from rats injected with glucose, the rate of increase of the FFA content of the medium after removal of the fat tissue was similar to the rate of increase in the presence of the tissue. If heparin was present in the medium, the rate of formation of FFA was greater initially and was maintained at a higher level when the fat tissue was removed.

When the fat tissue was obtained from fasted rats, FFA continued to be formed in the medium after removal of the tissue, but the rate of formation was reduced. Heparin had little effect on the rate of formation of FFA in these circumstances.

DISCUSSION

Activity of Fat Tissue from Animals Injected with Glucose. The experiments reported here show that when intact epididymal fat bodies from rats injected with glucose are incubated with chyle *in vitro*, the FFA formed in the incubation medium are derived from the chyle triglyceride by the action of an enzyme closely resembling the clearing factor lipase. Thus production of FFA is high at pH 8.1 but low at pH 6.0; and production is reduced in the presence of protamine sulfate, sodium pyrophosphate, and high concentrations of sodium chloride. Heparin, which increases the

TABLE 3. RATE OF APPEARANCE OF FFA IN THE MEDIUM FOLLOWING THE REMOVAL OF EPIDIDYMAL FAT TISSUE*

Fat Body Present (1st incubation period)			Fat Body Absent (2d incubation period)	
−Heparin	+Heparin		−Heparin	+Heparin
Glucose Injected				
2.5	6.0		1.82	6.85
4.5	7.8		4.8	8.1
Fasted 48 Hours				
0.70	0.75		0.38	0.43
0.47	0.60		0.37	0.20

* Rate of appearance expressed as μmoles/fat body/hr.

The fat tissue was incubated in either 6 ml. (glucose-injected rats) or 4 ml. (fasted rats) of a bovine albumin solution (100 mg./ml.) containing 7 μmoles of esterfied fatty acids per ml. as chyle. Heparin, if present, was at a final concentration of 0.25 units/ml. Samples of the medium (1 ml.) were taken (*a*) immediately after the introduction of the fat tissue, (*b*) following its removal 90 minutes later, and (*c*) after incubation of the medium for a further 90 minutes.

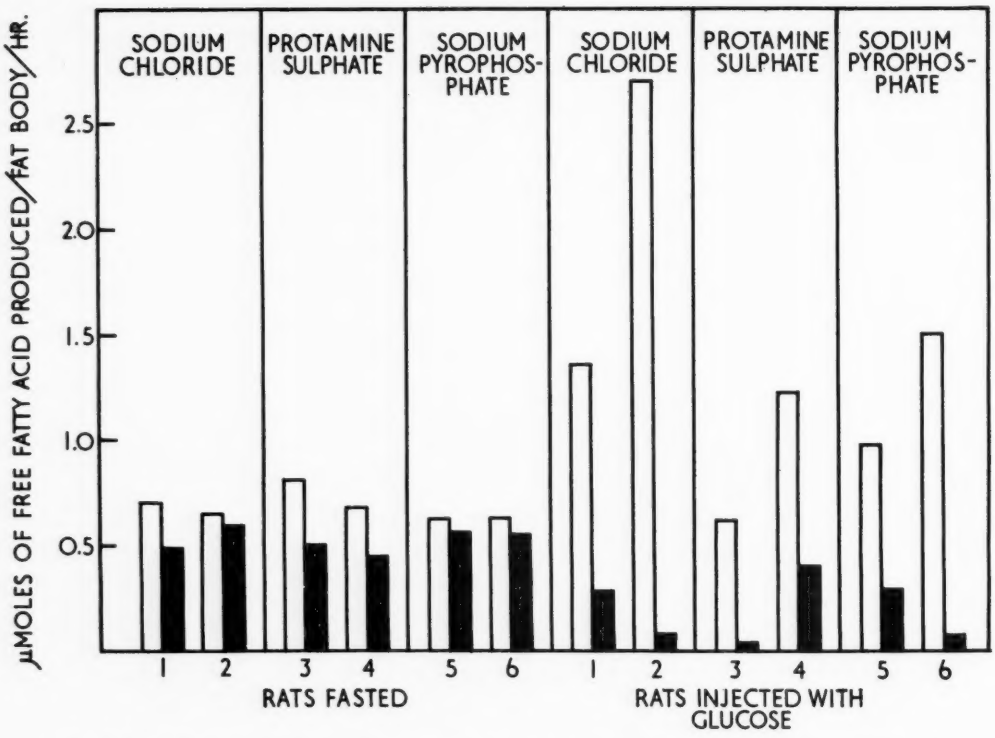

FIG. 3. The effect of various inhibitors on the appearance of FFA in the medium when epididymal fat bodies from rats either (*a*) fasted or (*b*) fed and injected with glucose were incubated with chyle and albumin *in vitro*. The final concentrations of the inhibitors were: sodium chloride, M; protamine sulfate, 5 mg./ml.; sodium pyrophosphate, 0.01 M. Production of FFA without inhibitor present (□); production of FFA with inhibitor present (■). Conditions of incubation as described in the Methods.

activity of the clearing factor lipase in extracts of adipose tissue, also increases the amount of FFA formed in these experiments.

Lipase is released from the tissue into the medium, both in the absence and presence of heparin, though the activity released is greatest when heparin is present. It seems probable that both the ability of heparin to release the clearing factor lipase from the tissues (16) and the affinity of the enzyme for its substrate (17) are factors controlling the appearance of enzyme activity in the medium.

These observations confirm and extend those reported recently by Hollenberg (1) and by Cherkes and Gordon (2). Hollenberg has shown that hydrolysis of the triglyceride component of a coconut oil emulsion by rat epididymal adipose tissue occurs *in vitro* when the fat tissue is obtained from rats fed their normal diet. He showed that heparin increased the lipolytic activity of the tissue and that lipase was released into the incubation medium. The activating effect of heparin could also be demonstrated with tissue from fasted rats, but only if glucose and insulin were present in the incubation medium. Hollenberg identified the lipase as the clearing factor lipase on the basis of the activating effect of heparin. Cherkes and Gordon (2), using the same fat tissue and a similar triglyceride substrate, demonstrated the ability of heparin to liberate lipase *in vitro* when the tissue was obtained from rats fed carbohydrate. They identified the lipase as the clearing factor lipase by the inhibitory effect of protamine sulfate and M sodium chloride solutions.

The demonstration of the activity of the clearing factor lipase in the adipose tissue of animals fed their normal diet or given carbohydrate in excess of their immediate nutritional requirements raises the question of the role of the clearing factor lipase in such circumstances. Elsewhere it has been suggested that the enzyme may be present normally in association with the walls of the blood vessels (18, 19) and that it may

act at this site to facilitate the passage of triglycerides from the blood to the tissues (16). It seems reasonable to suggest, therefore, that its activity in adipose tissue, under conditions that favor the deposition of triglyceride in the fat depots, may be concerned with the transport of triglyceride into this tissue.

Activity of Fat Tissue from Fasted Animals. During fasting the concentration of FFA in the blood rises (6, 20, 21, 22); and when epididymal fat tissue from fasted rats is incubated in an albumin medium *in vitro* in the absence of triglyceride substrate, FFA are released into the medium (7, 23). It has also been shown that when fat is mobilized following the injection of epinephrine, the proportion of mono- and diglycerides in adipose tissue is increased (24).

These observations have led to the view that under conditions in which the depot fat is mobilized, as, for example, during fasting, the depot triglyceride is hydrolyzed *in situ* and the FFA produced are carried in the blood from the depots to the tissues where they are utilized (25). In the present investigation, and in that of Hollenberg (1), however, triglycerides in the incubation medium were not hydrolyzed by epididymal fat tissue from fasted rats. Free fatty acids did appear in the medium in the present study, at a slow rate similar to that observed by Gordon and Cherkes (7) and by Reshef *et al.* (23), but their appearance was uninfluenced by the presence of chyle and was only slightly reduced when inhibitors of the clearing factor lipase were present and when the pH was reduced from 8.0 to 6.0. The rate of appearance of FFA was increased in the presence of heparin, but this effect was reduced when the period of fasting was raised from 18 to 48 hours. Thus no evidence has been obtained for a role of the clearing factor lipase in the release of FFA by epididymal fat tissue from fasted rats. If lipolysis of the depot triglyceride does occur *in situ* during fasting, either it is due to a lipase that is distinct from the clearing factor lipase (26, 27) or, if the clearing factor lipase is responsible, then this enzyme is no longer demonstrable by methods which can detect it in intact adipose tissue obtained from rats that have received glucose.

Limitations of the Method. In conclusion, certain limitations of the present type of study must be mentioned. First, changes in the composition of the incubation medium only are detectable. Changes in the composition of the fat tissue, that may or may not be reflected in changes in the medium, may also occur. Again, only net changes in the FFA content of the medium are determined. Both uptake and release of FFA by the fat tissue may take place under certain

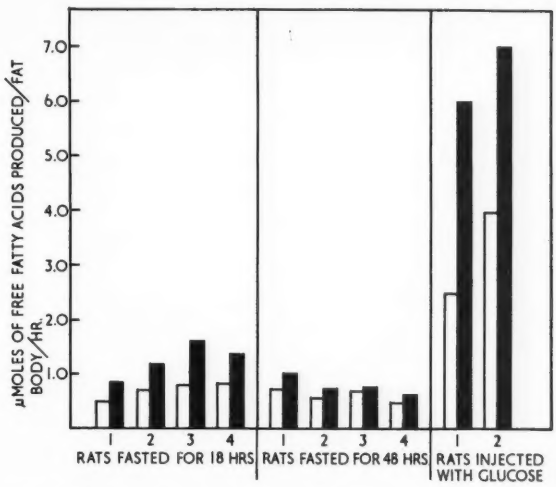

FIG. 4. The effect of heparin on the appearance of FFA in the medium when epididymal fat bodies from rats either (*a*) fasted or (*b*) fed and injected with glucose were incubated with chyle and albumin *in vitro*. A quantity of chyle containing 24 μmoles of esterfied fatty acid was added to 4 ml. of a solution containing 100 mg. of albumin/ml. (pH 8.1). Heparin was either absent (□) or present (■) at a final concentration of 0.25 units/ml. Samples (1 ml.) of the medium were taken immediately after the introduction of the fat tissue and after incubation for either 1 or 3 hours. The shorter period of incubation was used with fat bodies from the rats injected with glucose.

circumstances. Stern and Shapiro (28), for instance, using mesenteric fat tissue from rats starved for 5 days, reported an uptake of both triglyceride and FFA by the tissue *in vitro*.

Further, the experiments relate only to the two extreme nutritional states of complete fasting and of the intake of carbohydrate in excess. Some similar experiments were carried out on epididymal fat tissue obtained from rats fed their normal diet. The results suggested that hydrolysis of chylomicron triglyceride in the incubation medium occurred as a result of the action of the clearing factor lipase, but the extent of hydrolysis was less than that occurring with tissue obtained from animals injected with glucose, and the results were less clear cut. Glucose was not administered in the drinking water in this study because it was felt that the absorption of food might be thereby inhibited (29) and because the amount of glucose taken by an animal over a given time interval could not easily be controlled.

The present study only relates to intact epididymal fat tissue. Zemplényi and Grafnetter (30) have reported that when minced mesenteric fat is incubated with lipemic serum, the amount of FFA appearing in the medium is independent of whether the animals

from which the tissue was derived were fed their normal diet or were fasted for 60 hours.

Finally, it is evident that results obtained with systems similar to that studied here, in which the tissue is exposed only on its external surface to substances in the incubation medium, cannot be related directly to the situation in the intact animal. Adipose tissue *in vivo* has a rich blood supply, and the surface available for interaction and interchange between substances present in the blood and components in the tissue is very much greater than that in the system *in vitro*. For the same reason, neither triglycerides nor FFA are likely to accumulate *in vivo* in the blood in the concentrations studied here. The effect of such concentrations on the tissue and its metabolism is unknown.

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The influence of epinephrine and fasting on adipose tissue content and release of free fatty acids in obese-hyperglycemic and lean mice

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SUMMARY

Epididymal-fat-pad adipose tissue obtained from obese-hyperglycemic mice exhibited an impairment in its ability to mobilize free fatty acids (FFA) when incubated with epinephrine and in response to a 16-hour fast. Unexpectedly, FFA production by adipose tissue of fed obese mice was significantly greater than that observed in adipose tissue obtained from lean littermates. It is suggested that the impaired mobilization of FFA may be implicated in the etiology of this type of obesity.

Recent investigations have demonstrated several abnormalities in lipogenesis in obese-hyperglycemic mice. Thus, *in vivo* studies revealed an increased incorporation of C^{14}-labeled acetate into liver and carcass fatty acids even when obese mice were underfed (1, 2). Similarly, liver slices from obese-hyperglycemic mice exhibit an accelerated rate of lipogenesis from acetate *in vitro* (3). In addition, surviving adipose tissue from these obese animals has been reported to have a high rate of lipogenesis when acetate (4) or glucose (5) is used as substrate. Renold *et al.* (6) have recently found that while oxidation of glucose as well as its conversion to fatty acids, glycerol, and glycogen by adipose tissue was about half normal when expressed per mg. of nitrogen; these parameters were in excess of normal in terms of total tissue. Lipogenesis from acetate, on the other hand, was significantly enhanced even when tissue nitrogen was used as a reference standard for calculation (6).

These findings have placed considerable emphasis on accelerated lipogenesis as the factor responsible for the development and maintenance of this form of obesity, while relatively little attention has been paid to the possible contribution of mobilization of lipids from fat depots. On the basis of studies with C^{14}-labeled palmitic acid incorporated into the diet, Bates *et al.* (7) concluded that fat mobilization is impaired in the obese-hyperglycemic mouse. The present investigation was designed to explore this facet of the problem by comparing the rate of FFA release from adipose tissue of obese-hyperglycemic mice with that of their nonobese siblings in response to fasting and epinephrine stimulation *in vitro*.

MATERIAL AND METHODS

Obese-hyperglycemic mice in three different phases of obesity and consequently of three age groups and their lean siblings were used. Mice in the static phase of obesity (weight plateaued) were one year old at sacrifice and weighed 58 to 76 g. Their lean controls were in the 29 to 32 g. weight range. The dynamic group (rapid weight gain) of obese mice was 12 to 16 weeks old and weighed 41 to 54 g. in contrast to the lean mice weighing 22 to 30 g. The remaining group was comprised of obese mice in the onset phase of obesity, 5 to 7 weeks old, weighing 28 to 34 g., and their nonobese littermates weighing 18 to 24 g.

Portions (50 to 60 mg.) of epididymal-fat-pad adipose tissue from etherized obese mice and their lean littermates were incubated in a final volume of 1.0 ml. of Krebs-Ringer bicarbonate medium (8) containing 0.1 per cent glucose and 2 per cent bovine serum albumin. Incubation was accomplished at 37°C in an atmosphere of 5 per cent CO_2 and 95 per cent air in a Dubnoff metabolic shaker oscillating at 60 to 70 cycles

per minute. The FFA content of adipose tissue and release into the medium were measured by the method of Dole (9) 3 hours following the addition of epinephrine[1] at a final concentration of 1 μg. per ml. incubation medium or, in the case of the controls, an equal volume of distilled H_2O. Unless otherwise indicated, the mice were subjected to an overnight fast of approximately 16 hours prior to incubation of tissue.

Nitrogen content of adipose tissue was determined by nesslerization (10) on portions of fat-extracted tissue obtained from areas contiguous to fragments incubated for FFA determinations.

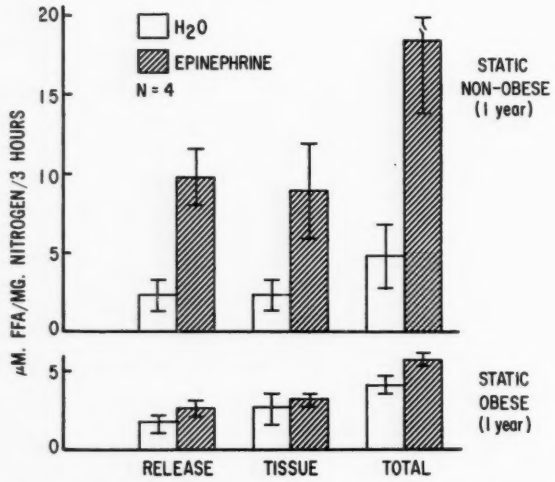

FIG. 1. The influence of epinephrine on adipose tissue content and release of FFA in obese-hyperglycemic mice during static phase of obesity and in their lean littermates.

RESULTS

Static Obesity. Adipose tissue from the fasted, older obese-hyperglycemic mice and from their lean littermates produces approximately equivalent amounts of FFA during incubation without hormonal stimulation. In the nonobese group, epinephrine induces a three- to fourfold increase in the FFA content of tissue, that released to medium, and consequently total production (Fig. 1). Although adipose tissue from the obese-hyperglycemic mice is also influenced by the lipolytic action of epinephrine (Fig. 1), it is relatively insensitive, e.g., the epinephrine effect is only one-ninth that observed for the nonobese group ($p < .01$).

Onset Obesity. The results obtained with adipose tissue from young obese-hyperglycemic mice, which were gaining weight rapidly, and from their lean littermates, are represented in Figure 2. Again FFA levels in the unstimulated tissues are the same and a lipolytic effect of epinephrine is apparent in both nonobese and obese mice; however, in the latter, the epinephrine effect is significantly diminished ($p < .01$).

Effect of Fasting. The influence of fasting on mobilization of FFA was investigated in a group of obese-hyperglycemic mice in the dynamic phase of obesity and their lean siblings. Fed mice were allowed chow pellets ad libitum. The results are tabulated in Table 1. As expected, nonobese mice show an increase over the baseline release of FFA when fasted ($p < .02$). The total FFA production was of borderline significance ($p = .06$), and probably would be more impressive if more animals were studied. By contrast, the obese mice do not increase the production of FFA to any significant extent during the fast ($p < .1$). However, the rate of fatty acid production by the adipose tissue of the fed obese animals is comparable to that in the fasted lean mice, although the difference between the levels in the fed and fasted obese mice is of questionable significance ($p < .06$). Since this point may be of considerable importance, another series of six lean and obese mice were compared during the fed state. Total production of FFA by the lean mice was 2.1 $\pm$ 0.32 μM FFA per mg. N and by the obese 3.6 $\pm$ 0.39 μM FFA per mg. N. This difference is significant ($p < .02$). As already observed with fasted onset and static phase obese mice, epinephrine exerts a lesser lipolytic effect on adipose tissue of dynamic

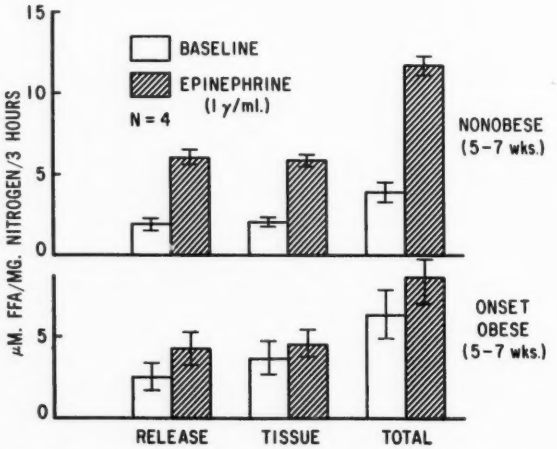

FIG. 2. The influence of epinephrine on adipose tissue content and release of FFA in obese-hyperglycemic mice during onset phase of obesity and in their lean littermates.

[1] Adrenalin®, Parke, Davis and Company, Detroit, Mich.

TABLE 1. THE INFLUENCE OF EPINEPHRINE AND OF FASTING ON ADIPOSE TISSUE CONTENT AND RELEASE OF FFA IN OBESE-HYPERGLYCEMIC MICE DURING THE DYNAMIC PHASE OF OBESITY AND IN THEIR LEAN LITTERMATES

	No./Grp.	Fed		Fasted	
		μM FFA*/Mg. Tissue Nitrogen/3 Hours			
		Baseline	Epinephrine	Baseline	Epinephrine
Lean	4				
Release		1.1 ± 0.37	8.0 ± 0.95	2.9 ± 0.39	6.1 ± 0.78
Tissue		1.5 ± 0.25	8.1 ± 0.46	2.0 ± 0.52	7.1 ± 0.73
Total		2.6 ± 0.62	16.1 ± 0.79	4.9 ± 0.83	13.2 ± 1.37
Obese	4				
Release		2.1 ± 0.29	3.4 ± 0.40	2.3 ± 0.51	4.3 ± 0.47
Tissue		2.4 ± 0.33	3.4 ± 0.12	2.7 ± 0.50	3.5 ± 0.24
Total		4.5 ± 0.62	6.8 ± 0.46	5.0 ± 0.58	7.8 ± 0.48

* Mean ± standard error.

phase obese mice both when fed and during fasting than it does in their lean siblings (Table 1).

Nitrogen Content of Adipose Tissue. The data obtained from the nitrogen content of adipose tissue are summarized in Table 2.

TABLE 2. MG. NITROGEN/100 MG. FRESH ADIPOSE TISSUE O-H MICE AND LEAN SIBLINGS

Age	Nonobese*	Obese*
5–7 weeks†	.402 ± .01	.130 ± .01
12–16 weeks‡	.255 ± .01	.142 ± .01
1 Year§	.198 ± .01	.209 ± .02

* Mean ± standard error.
† Onset obesity.
‡ Dynamic obesity.
§ Static obesity.

During the onset phase of obesity, the nitrogen content of adipose tissue from obese-hyperglycemic mice is only 30 per cent of that found for the nonobese ($p < .001$). As the mice become older, the disparity between obese and nonobese mice tends to disappear, e.g., in the dynamic group, adipose tissue from obese mice contains 80 per cent as much as their lean siblings ($p < .001$). However, during the static phase of obesity, there is no statistically significant difference between the two groups ($p < .1$). In the obese group, there is no significant difference in adipose tissue nitrogen content between onset and dynamic phases of obesity; however, the difference between static, on the one hand, and onset and dynamic, on the other, is significant at the 1 per cent level. As regards the 5-to-7-week-old nonobese mice, the nitrogen content of adipose tissue is considerably greater than that observed for the two older groups ($p < .001$). The difference between the 12-to-16-week group and the 1-year group is also of statistical significance ($p < .02$).

DISCUSSION

The results of these experiments indicate that in addition to the defects in lipogenesis reported for the obese-hyperglycemic mice, these animals also differ from their normal siblings in their ability to mobilize fatty acids from adipose tissue in response to fasting and epinephrine. In a glucose-containing medium, the lesser net mobilization of FFA by the obese adipose tissue could be due either to a lower rate of lipolysis or to an increased rate of reesterification or to both. Unexpectedly, FFA production from adipose tissue of fed mice in the dynamic phase of obesity was slightly, but significantly, greater than in the controls, being comparable to that of lean mice at the end of a 16-hour fast. Fatty acid production was depressed in adipose tissue from fed lean mice. Perlman *et al.* (11) have recently reported that the FFA content of adipose tissue increases during prolonged fasting in lean but not in obese-hyperglycemic mice. Our data are in agreement, although a shorter fast was employed. The significance of the slightly greater release of fatty acids in the fed obese animals is uncertain, but the inability of these mice to increase fatty acid production during fasting may be of significance in the etiology of this type of obesity.

The failure of the adipose tissue of mice during any phase in the development of obesity to respond to epinephrine to a degree comparable to that exhibited by the lean controls is even more significant. There is now much evidence to suggest that the catecholamine hormones play a significant role in the tonic control of fatty acid mobilization (12, 13). Wertheimer *et al.* (14) have made the interesting observation that Dibenzyline® blocks the accelerated release of FFA from adipose tissue of rats which have been fasted, exposed to cold, treated with endotoxin, made hyperthyroid with triodothyronine, or are diabetic. The impaired lipolytic response of the obese mice to epinephrine may thus represent a major defect in the energy

economy of this animal. The inability of the obese-hyperglycemic mouse to withstand hypothermia and to increase its oxygen consumption when exposed to cold (15) might be another reflection of this defect. Finally, it might be noted that Bogdonoff[2] has recently found that certain obese patients have an impaired plasma FFA response to norepinephrine infusion.

Because of the difference in the thickness and consistency of the fat pads of the obese mice compared to their lean siblings, it might be argued that the diminished lipolytic response to epinephrine is due to a relative inability of the hormone to penetrate the tissue of the obese mice. The difference in response of adipose tissue to fasting in the obese and lean mice would seem to minimize this factor.

The decreasing difference in adipose tissue nitrogen content between obese and nonobese mice with increasing age presumably appears to be related not so much to a decrease in percentage of fat content of obese adipose tissue as to the increase in fat content of nonobese adipose tissue. Apparently the fat content of adipose tissue in the lean group increases with age, while at the same time it is possible that there is a hypertrophy or hyperplasia of adipose tissue, or both, with increasing age in the obese group.

[2] Personal communication from M. D. Bogdonoff.

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Effect of infusions of phosphatides upon the atherosclerotic aorta *in situ* and as an ocular aortic implant*

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SUMMARY

Rabbits fed cholesterol- and oil-supplemented diet were carefully paired for equality of plasma cholesterol concentration during the feeding period and a subsequent 30 days of normal diet. The normal diet was continued and one of each pair of rabbits was then subjected to intravenous infusion of mixed phosphatides of animal or soybean origin twice weekly for an average of 11 infusions. At the end of this time the aortas of the infused rabbits showed markedly less atherosclerosis than their paired controls, both grossly and when assessed on the basis of cholesterol content. When implants of atherosclerotic aorta were made into the anterior chamber of the eyes of normal host rabbits, infusions of phosphatide did not accelerate the decline in cholesterol content of the implants or affect the normal cholesterol content of the host rabbit's own aorta. When normal rabbit aorta was implanted into normal host rabbits thereafter fed a diet enriched in cholesterol and oil, infusion of phosphatide had no effect on atherosclerosis of the host's own aorta. However, a marked increase was observed in sudanophilia and cholesterol content of the aortic implants in those rabbits receiving infusions. Apparently the lipid dynamics of ocular aortic implants differs from that of the aorta *in situ*. Infusions of phosphatide can alleviate atherosclerosis when a cholesterol-free dietary is followed but may worsen atherosclerosis when a cholesterol-free diet is not followed.

The sustained infusion of various phosphatides has been observed (1, 2) to elevate promptly the level of plasma cholesterol. One source of this cholesterol appears to be the cholesterol already present in various extravascular tissues (3). This suggests that under proper circumstances, experimental elevation of plasma phospholipid content leads to a loss of cholesterol from various tissues to the blood.

These findings raised the possibility that chronic infusion of the atherosclerotic rabbit with a suitable mixture of phosphatides might lead to a removal of some of the cholesterol present in the atherosclerotic plaques of the animal. Accordingly, a series of atherosclerotic rabbits was infused with a relatively crude mixture of phosphatides derived from animal brain. The results (4) suggested that such infusions indeed led to a diminution in both the size and the cholesterol content of previously formed atherosclerotic infiltrations. In view of these results, this phenomenon was investigated more extensively, employing relatively pure suspensions of "lecithin" derived from vegetable sources.

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METHODS

The Infusion of Various Phosphatide Suspensions into Atherosclerotic Rabbits. Three separate groups of young male rabbits were fed a Wayne rabbit chow diet enriched with 2 per cent cholesterol and 2 per cent cottonseed oil for 3 months. They were then returned to a diet of only Wayne rabbit chow for a period of not less than 30 days. Heparinized plasma for analysis of cholesterol content was obtained monthly from these rabbits while on the enriched diet, and again 30 days after their return to the Wayne chow diet. Rabbits were paired as experimental and control when found to have similar plasma cholesterol levels during the cholesterol feeding period and also the same plasma

cholesterol value 30 days after cessation of cholesterol feeding. It should be stressed that in our experience, if a pair of rabbits is desired that exhibits the same amount of aortic atherosclerosis, not only should their average plasma cholesterol content during the cholesterol feeding be essentially similar, but also the "fall-off" level 30 days after cessation of feeding should be the same. Thus if one of a pair of rabbits exhibits the same average plasma cholesterol as its mate during the cholesterol feeding period, but remains

TABLE 1. EFFECT OF INFUSION OF VARIOUS PHOSPHATIDES UPON THE ATHEROSCLEROTIC AORTA OF THE RABBIT

No. of Rabbits	Average Weight	Average Plasma Cholesterol (mg./100 ml.)*		No. of Infusions	Aorta		
		During Cholesterol Feeding	One Mo. After Cholesterol Feeding		Degree of Atherosclerosis	Cholesterol	Total Lipid
	g.				*0–5*	*g./100 g.*	*g./100 g.*
A. Rabbits Infused with Crude Phosphatide (Animal Brain)							
Infused–6	2867	995 *802–1270*	167 *71–404*	8 *5–16*	3.3 *2.0–5.0*	6.69 *1.1–14.2*	34 *19.5–42.3*
Control–6	3087	958 *738–1174*	213 *91–485*	8† *5–16*	4.8 *4.5–5.0*	12.8 *9.5–17.2*	38.0 *27.2–49.2*
B. Rabbits Infused with Soybean Phosphatide (Sterling-Winthrop)							
Infused–7	3373	585 *241–777*	203 *41–420*	13 *12–13*	2.7 *1.5–4.0*	4.18 *1.3–6.9*	—
Control–7	3164	568 *489–1277*	187 *26–104*	0	4.5 *3.5–5.0*	5.31 *1.97–7.11*	—
C. Rabbits Infused with Soybean Phosphatide (Cutter)							
Infused–6	3176	909 *440–1333*	68 *23–104*	12 *11–12*	2.3 *1.0–3.0*	5.04 *3.45–7.1*	—
Control–6	2076	908 *489–1277*	72 *26–104*	0	4.0 *2.0–5.0*	6.49 *2.74–9.9*	—
Totals							
Infused–19	3151	822	149	11	2.7	5.34‡	—
Control–19	2796	799	159	—	4.6	8.05‡	—

Figures in italics indicate range.

* Cholesterol concentrations prior to infusion are included to show equality of exposure to atherosclerogenic infiltration.

† Infusions of dextrose solution (5%).

‡ t value equals 3.4.

significantly higher 30 days after its removal from the cholesterol diet, almost invariably such a rabbit exhibits far more aortic atherosclerosis than its paired mate. In other words, the rate of decline of a previously high plasma cholesterol is inversely proportional to the amount of excess cholesterol stored in the animal's tissues, including its aorta, during the prior cholesterol feeding (5).

After the rabbits of each group were paired, one of each pair was subjected to a series of 5 to 16 intravenous infusions of various phosphatide suspensions maintained for 6 hours and given twice a week. Plasma samples were obtained usually before and at the end of each infusion period and analyzed for cholesterol and phospholipid (4). At the end of the series of infusions, the infused and its paired control rabbit were sacrificed. The aorta was exposed, inspected grossly, and graded on a scale from 0 to 5 (4). Then a section of the aorta extending 10 cm. from the insertion of the semilunar valves was obtained routinely from both the infused and control rabbits and analyzed for its cholesterol content (4).

Group A consisted of six pairs of rabbits. One of each pair received 5 to 16 infusions of a crude mixture of phosphatides derived from animal brain.[1] Approximately 60 to 80 ml. of a 5 per cent suspension in 5 per cent dextrose solution was infused over the 6-hour period. The paired control rabbits received similar infusions of only 5 per cent dextrose. Little or no adverse reaction was observed during or after the infusions. The phosphatide mixture was analyzed for both its sterol and phospholipid content (2).

Group B consisted of seven pairs of rabbits. One of each pair received 12 to 13 6-hour infusions of a mixture of phosphatides derived from soybean (Sterling-Winthrop) similarly made up to 5 per cent and suspended in 5 per cent dextrose solution; the control rabbits were not infused.

Group C consisted of six pairs of rabbits treated exactly like those of Group B except that one of each pair received a mixture of phosphatides derived from soybean obtained from a different manufacturing source (Cutter Laboratories, Inc.).

The Infusion of Various Phosphatide Suspensions into Normal Rabbits Bearing Corneal Atherosclerotic Aortic Implants. Three different groups of normal rabbits were employed in this experiment. Three closely similar segments of the atherosclerotic aorta of a rabbit previously fed cholesterol were implanted into the anterior eye chamber of pairs of rabbits of each group, according to the Higginbotham technique (6). Then one of each pair of rabbits was subjected to a series of 4 to 13 infusions of the same phosphatide mixtures employed above. One of each pair of Group A rabbits (five pairs) received infusions of the animal brain phosphatide.[2] One of each pair of Group B rabbits (21 pairs) was infused with the purified "lecithin" prepared by Sterling-Winthrop. One of each pair of Group C rabbits (six pairs) was infused with the Cutter-prepared "lecithin" suspension. At the end of the series of infusions, the rabbits were sacrificed, the implants removed, a small section removed for Sudan IV staining, and the remainder analyzed for cholesterol content according to previously described methods (7). In five pairs of rabbits of Group B a 5-cm. section of each rabbit's own aorta was removed and analyzed for cholesterol.

The Infusion of a "Lecithin" Suspension into Rabbits Bearing Corneal Aortic Implants During the Ingestion of a Cholesterol and Oil-Enriched Diet. Three segments of a normal rabbit's aorta were implanted into the anterior eye chamber of each of 10 normal rabbits. One week later the 10 rabbits were allowed to ingest a diet enriched with 2 per cent cholesterol and 2 per cent cottonseed oil for 30 days. In addition, 5 of the 10 rabbits were infused for 8 hours every 4 days, for a total of 8 infusions, with the purified soybean "lecithin" suspension prepared by Sterling-Winthrop. Blood samples obtained at the beginning and at the end of the second, third, and fourth weeks were analyzed for plasma total cholesterol and phospholipid. At the end of the 30-day feeding period all rabbits were sacrificed, and the aortic implants were removed and studied as described above. In addition, each rabbit's own aorta was inspected and graded, and a 10-cm. segment taken for cholesterol analysis.

RESULTS

The Effect of Phosphatide Infusions on Aortic Infiltration of Atherosclerotic Rabbits. Following each infusion of "lecithin" derived from either animal or vegetable sources, the plasma phospholipid and cholesterol promptly rose, as described previously (1, 2). Thus in a typical infusion of the six rabbits of Group A, the average serum cholesterol and phospholipid rose in 6 hours from 42 and 92 mg. per 100 ml., respectively, to 110 and 857 mg. per 100 ml. These lipid elevations usually disappeared 24 to 48 hours after each infusion. There was no discernible tendency for the plasma cholesterol response to increase or decrease

[1] "Ninety per cent pure" animal "lecithin," Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] See footnote 1.

with repeated phosphatide injections, nor did the initial injection provoke a markedly different response than did subsequent injections.

The aortas of the atherosclerotic rabbits given repeated infusions of "lecithin" suspensions, whether derived from animal or vegetable sources, exhibited markedly less gross atherosclerosis than their paired controls (Table 1). Thus the average gross degree of aortic atherosclerosis in the 19 treated rabbits was adjudged as 2.7, and that of the 19 controls, 4.6. Upon analysis, the average cholesterol content of the aortas of the treated animals also was less, being 5.34 g. per

TABLE 2. EFFECT OF INFUSIONS OF VARIOUS PHOSPHATIDES UPON THE OCULAR-IMPLANTED ATHEROSCLEROTIC AORTA OF THE RABBIT

No. of Rabbits	Average Weight	No. of Infusions	Implantation	Aorta Implant			Aorta *in Situ*
				Initial Cholesterol Content	Terminal Cholesterol Content	Presence of Terminal Sudanophilia	Cholesterol
	g.		*days*	*g./100 g.*	*g./100 g.*	*per cent*	*g./100 g.*
A. Rabbits Infused with Crude Phosphatide (Animal Brain)							
Infused–5	3124	9 *8–10*	49 *42–57*	6.97*	3.59 *2.35–4.78*	80	—
Control–5	3062	0	49 *42–57*	6.97*	3.12 *1.31–5.56*	80	—
B. Rabbits Infused with Soybean Phosphatide (Sterling-Winthrop)							
Infused–21	3061	9 *3–13*	43 *22–67*	7.95 *5.13–12.9*	4.06 *0.99–6.7*	76	0.419 *0.388–0.496*
Control–21	2954	0	43 *22–67*	7.95 *5.13–12.9*	3.92 *0.6–9.34*	71	0.396 *0.37–0.427*
C. Rabbits Infused with Soybean Phosphatide (Cutter)							
Infused–6	2784	6 *3–7*	41 *32–44*	5.37 *4.59–6.17*	1.85 *1.34–3.03*	0	—
Control–6	2890	0	41 *32–44*	5.37 *4.59–6.17*	2.17 *1.11–4.50*	0	—
Average of All							
Infused–32	3019	8	44	7.31	3.57	62	
Control–32	2959	0	44	7.34	3.47	59	

Figures in italics indicate range.

* All implants taken from single area of aorta; therefore no range of cholesterol content.

100 g. of dry weight as compared to 8.05 g. per 100 g. of dry weight of the control aortas—a significant difference ($t = 3.4$). The aortic total lipid content, however, of the infused rabbits of Group A failed to exhibit a significant decline.

The Effect of Phosphatide Infusions upon Corneal Atherosclerotic Aortic Implants. The expected decline (7) in the cholesterol content and sudanophilia of atherosclerotic aortic implants many weeks after their deposition was observed once more in both the infused and the control rabbits. However, this disappearance, as Table 2 clearly depicts, was not accelerated by the infusion of any of the three types of phosphatide suspensions. It is of interest also that the cholesterol content of the normal aorta of the host animal (Table 2, B) was not significantly altered by as many as 10 infusions of phosphatide.

The Effect of Phosphatide Infusions upon the Cholesterol Infiltration of the Aorta and Corneal Implant of Cholesterol-Fed Rabbits. The five rabbits ingesting excess cholesterol for 4 weeks, during which time they also received 7 infusions of phosphatide, failed to exhibit any significant change in either the gross degree of aortic atherosclerosis or in the aortic cholesterol content as compared to the controls (Table 3). However, a marked change was observed in the corneal implants. Of the implants of the infused rabbits, 40 per cent exhibited some sudanophilia as compared to 7 per cent in the controls. Similarly, the average cholesterol content of the implants in the infused animals was over twice as much as was found in the controls.

DISCUSSION

In earlier studies (2, 3) the hypercholesterolemic effects of various phosphatide infusions were seemingly due to the mobilization and entrance into the blood of already formed cholesterol present in various tissues. There was little or no evidence that such infusions altered the endogenous rate of cholesterol synthesis. For example, prior deprivation of the animal from dietary cholesterol (a procedure supposedly capable of increasing the rate of endogenous cholesterol synthesis) tended to reduce the hypercholesterolemic effect usually attained during phosphatide infusion. Conversely, prefeeding of cholesterol heightened the hypercholesterolemic effect of such infusion. The results obtained in the present study are in general agreement with these earlier findings. However, work now in progress in this laboratory indicates that the hyper-

TABLE 3. EFFECT OF INFUSION OF SOYBEAN PHOSPHATIDES UPON THE OCULAR-IMPLANTED AORTA AND AORTA *in Situ* OF THE CHOLESTEROL-FED RABBIT

No. of Rabbits	Average Weight	Average Plasma Cholesterol*	Average Plasma Phospholipid*	Aorta Implant†		Aorta *in Situ*	
				Sudanophilia	Cholesterol	Aver. Degree Atherosclerosis	Cholesterol
	g.	*mg./100 ml.*	*mg./100 ml.*	*per cent*	*g./100 g.*	*0–5*	*g./100 g.*
A. Cholesterol-Fed Rabbits Infused with Soybean Phosphatides							
5	3053	842	385	40	5.05	0	0.89
Standard error		±26.8	±20.6	—	±1.3		±0.14
B. Control Cholesterol-Fed Rabbits							
5	2705	447	264	7	2.12	0	0.81
Standard error		±101	±39	—	±0.35		±0.1

* Represents average of plasma analyses at beginning, second, third, and final week of feeding period.
† Original cholesterol content of aorta implant was 0.91 g./100 g. of dry weight.

cholesterolemic effect of phosphatide infusion into normally fed animals may be diminished, though not abolished, by administration of a drug that strongly inhibits cholesterol synthesis.

The infusion of phosphatides of either animal or vegetable source into the normocholesterolemic but atherosclerotic rabbit ingesting a cholesterol-free diet invariably induced a temporary hypercholesterolemia during such infusions. Following a series of such infusions, the aortas of such animals exhibited far less atherosclerosis and significantly less cholesterol than the untreated controls. It is our belief that during each infusion, some of the cholesterol making up the hypercholesterolemia observed was extracted from aortic atherosclerotic depots.

In earlier studies (8) we have called attention to the quantitatively different sequence of lipid dynamics that takes place in both the normal and atherosclerotic ocular-implanted aorta, as compared to the aorta *in situ*. First, a far greater degree of lipid infiltration takes place in the ocular-implanted aorta than in the animal's own aorta when this animal is fed excess cholesterol. Second, when the animal is removed from a high cholesterol diet, the lipid infiltration rapidly diminishes in the ocular implant but remains unchanged in the aorta *in situ*. This rapid disappearance of lipid from the ocular-implanted atherosclerotic aorta could not be hastened by the present series of phosphatide infusions.

When animals implanted with ocular fragments of normal aorta were placed upon a high cholesterol diet and also given frequent infusions of phosphatide suspensions, such animals, as was expected (1, 2), exhibited a greater degree of hypercholesterolemia than their uninfused controls. The aortas of both the infused and control animals, when examined 30 days later, failed to show either gross atherosclerosis or any excess cholesterol in the aorta *in situ*. However, again as expected (8), the ocular implants already were heavily impregnated with cholesterol and were sudanophilic. It was of great interest that the cholesterol content and sudanophilia of the implants of the infused animals were far greater than those of the controls. Apparently the infusion of phosphatide simultaneously with the ingestion of excess cholesterol, by inducing a greater degree of hypercholesterolemia, led to a greater cholesterol deposit in the ocular-implanted aorta.

These latter findings appear to us to suggest two things: First, that the lipid dynamics of the implanted aorta and that of the aorta *in situ* are different, and one is not necessarily an index of the other's processes; second, the possibility that phosphatide infusions carried on at the same time that excess cholesterol is taken in the diet may, by the ability to maintain or prolong hypercholesterolemia derived from this dietary cholesterol, actually worsen, rather than alleviate, cholesterol deposition in various tissues. In this sense, then, phosphatide, as we suggested earlier (3), may act as a double-edged sword. Certainly the infusion of phosphatide into human subjects ingesting or absorbing large amounts of cholesterol might be expected to induce a hypercholesterolemia derived in part not from tissue depots of cholesterol but from dietary sources. If this is the case, its infusion might have deleterious rather than therapeutic effects.

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Notes on Methodology

Rapid ultramicro estimation of serum total cholesterol*

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SUMMARY

An ultramicro cholesterol technique based upon a new color reaction and requiring only 10 μl. of serum is proposed. The need for extraction with lipid solvent is eliminated by drying serum on filter paper segments and eluting directly in the ferrous sulfate color reagent. This method has been successfully applied to sera obtained from 200 blood donors.

► In 1934 Schoenheimer and Sperry (1) described a microcholesterol method using the Liebermann-Burchard reaction which required 0.2 ml. of serum. Subsequently there has been a tendency toward the use of smaller blood specimens for cholesterol analysis. Recently Galloway *et al.* (2) modified the macro method of Sperry and Webb (3) to measure cholesterol in as little as 40 μl. of serum. Zlatkis *et al.* (4) introduced a new ferric chloride-cholesterol color reaction which is also sufficiently sensitive for estimation of this lipid fraction in small quantities of serum. A simple micro technique based on this color reaction which employs only 0.1 ml. of serum has been described by Zak (5). Rosenthal and Jud (6) have further modified this procedure for use with 50 μl. of serum. It is the purpose of this report to describe a method for cholesterol quantitation which utilizes even smaller amounts of blood. The ferrous sulfate color reaction previously described by Searcy and Bergquist (7) has been applied to 10 μl. of serum. This makes possible accurate quantitation of cholesterol on ultramicro quantities of serum, thereby conserving blood for additional laboratory determinations.

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METHODS

Measurement of Serum Total Cholesterol. An aliquot of 10 μl. of serum is quantitatively applied to a 2 cm. square of lipid-free filter paper (Whatman #1). To prevent serum loss during the drying process, the filter paper segments are supported by two glass stirring rods. Depending on room temperature and humidity, a 10-to-15-minute period is usually sufficient to dry serum spots. The filter paper segments are folded and placed in the bottom of small test tubes containing exactly 2.0 ml. of a saturated solution of ferrous sulfate in glacial acetic acid. The total cholesterol is eluted by allowing the serum-spotted strips to remain in the color reagent for at least 1 hour. Exactly 1.5 ml. of the cholesterol-ferrous sulfate reagent mixture, which represents 7.5 μl. of serum, is then transferred to a Coleman microcuvette (10 × 75 mm.) and mixed with 0.5 ml. of concentrated sulfuric acid. A small stirring rod may be used to effect uniformity. The salmon-pink color is allowed to develop for at least 10 minutes. The tubes are placed in a microcuvette adapter and the optical densities are measured at 490 mμ. against a reagent blank with a Coleman Junior spectrophotometer. The reagent blank is prepared by eluting unspotted filter paper with the color reagent. The standard cholesterol is prepared in the ferrous sulfate color reagent.

RESULTS AND DISCUSSION

Color Production with Increasing Cholesterol Concentrations. Amounts of cholesterol ranging from 10 to 60 μg. in 1.5 ml. of ferrous sulfate color reagent were mixed with 0.5 ml. of concentrated sulfuric acid. After 10 minutes the color intensity was measured against a reagent blank. Figure 1 demonstrates that the chromogenic response followed Beer's law with the cholesterol concentrations employed. The sensitivity of the color reaction makes practical the quantitation of total cholesterol in as little as 10 μl. of serum.

Serum Cholesterol Extraction Efficiency. Aliquots of 10 μl. of a pooled serum were applied to filter paper and placed in the ferrous sulfate color reagent or in chloroform-methanol (2:1, v/v) for 1 to 60 minutes. The amount of cholesterol was quantitated directly in the ferrous sulfate extracts but the chloroform-methanol eluates were taken to dryness prior to treatment with the color reagent. Figure 2 demonstrates that in 6 minutes each mixture contained some 60 per cent of the total cholesterol. However, with the glacial acetic acid reagent, extraction proceeds more rapidly after

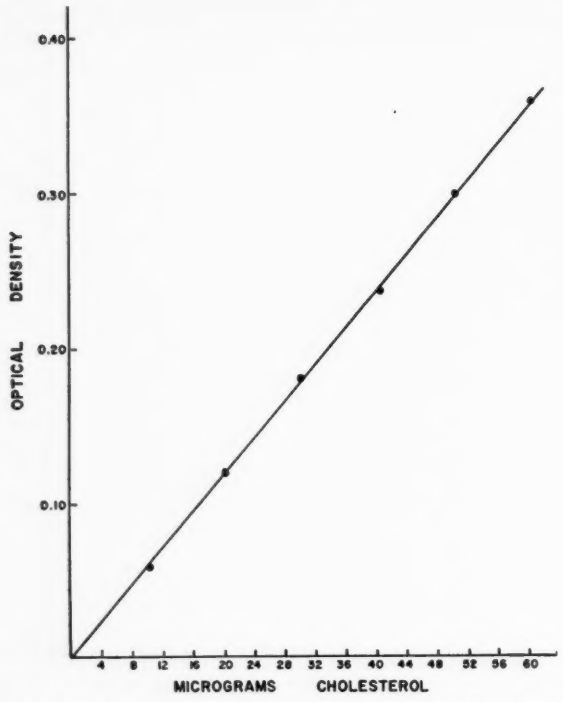

FIG. 1. Color production with increasing amounts of cholesterol treated with the $FeSO_4$ color reagent.

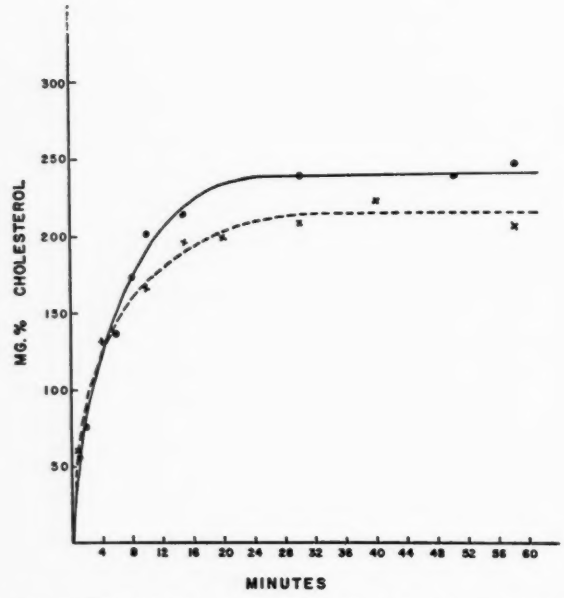

FIG. 2. Effect of time upon the extraction of cholesterol from serum applied to filter paper using $FeSO_4$ color reagent (———) and chloroform-methanol (------).

this time and appears complete in 40 minutes. No appreciable change in cholesterol values was noted up to 16 hours of elution. In contrast, chloroform-methanol (2:1, v/v) failed to extract the total cholesterol during the time intervals studied.

Recoveries of Steroid Chromogen. Aliquots of a pooled serum were placed on filter paper and allowed to dry in air at room temperature from 15 minutes to 13 days. They were then extracted with the ferrous sulfate color reagent. During the first 24 hours after serum application, no significant reduction in steroid chromogen could be observed (Table 1). However, exposure for 6 to 13 days resulted in approximately a 10 per cent loss of chromogen as compared to the first 24-hour measurement.

TABLE 1. RECOVERY OF STEROID CHROMOGEN FROM SERUM APPLIED TO FILTER PAPER

Storage Time	Total Cholesterol	Storage Time	Total Cholesterol
	mg./100 ml.		*mg./100 ml.*
15 minutes	249*	6 hours	255
1 hour	251	24 hours	250
2 hours	253	6 days	221
4 hours	243	13 days	229

* Each figure represents the average of at least three total cholesterol determinations.

A 10 μl.-aliquot of pooled serum having an average total cholesterol of 240 mg. per 100 ml. (7, 8) was applied to filter paper segments. Increasing amounts of a standard solution containing 200 mg. per 100 ml. cholesterol were placed on serum spots so that total cholesterols ranged from 320 to 440 mg. per 100 ml. The filter paper segments were dried and then eluted for 60 minutes in the ferrous sulfate reagent. The cholesterol recoveries shown in Table 2 range from 92 to 101 per cent of the theoretical values. The mean cholesterol recovery at the four concentrations was 95 per cent or above. These figures agree well with those reported by other workers (9) utilizing microcholesterol methods.

Reproducibility of the Method. Total cholesterol determinations were carried out in duplicate on 24 randomly selected sera from normal individuals (Table 3). The mean total cholesterol of these sera ranged

from 112 to 231 mg. per 100 ml. The differences between duplicates varied from 0 to 13 mg. per 100 ml. Expressed as a per cent of the mean, the variation averaged a little more than 3 per cent. These results compare favorably with those obtained by Clayton *et al.* (9) who report measurements of six aliquots of the same serum to be within 4.3 per cent of the highest cholesterol value.

Comparison of Ultramicro Technique with the Zak Cholesterol Method. Single cholesterol determinations were carried out on 42 randomly selected normal sera utilizing the proposed technique. Two-tenths ml. of the same sera was extracted with acetone-ethanol and quantitated, using the ferric chloride color reaction (8). Results of this study are illustrated in Figure 3. The solid line represents the theoretical relationship of cholesterol concentrations by the two methods. The actual cholesterol measurements by the macro and ultramicro techniques demonstrate a variation within acceptable limits.

Normal Values Obtained with the Method. Blood was collected from 200 male and female hospital blood donors. The serum was isolated and the total cholesterol was analyzed in 10 μl. aliquots. The normal values ranged from 122 to 350 mg. per 100 ml., with an average of 223 mg. per 100 ml.

TABLE 2. RECOVERY OF STEROID CHROMOGEN FROM CHOLESTEROL AND SERUM APPLIED TO FILTER PAPER

Cholesterol Present	Cholesterol Added	Total Cholesterol Found	Recovery
mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*per cent*
240	80	322, 314, 303	101, 98, 96
240	120	357, 354, 344	99, 98, 96
240	160	385, 395, 374	96, 99, 93
240	200	437, 416, 406	99, 95, 92

TABLE 3. REPRODUCIBILITY OF ULTRAMICROMETHOD FOR SERUM TOTAL CHOLESTEROL

Duplicates		Difference	Duplicates		Difference
mg./100 ml.	*mg./100 ml.*	*per cent of mean*	*mg./100 ml.*	*mg./100 ml.*	*per cent of mean*
198	208	4.9	146	150	2.7
188	198	5.2	160	163	1.9
208	208	0	187	184	1.6
167	172	2.9	208	205	1.5
110	114	3.6	188	200	3.1
229	232	1.3	175	178	1.7
208	218	2.1	140	146	4.2
168	166	1.2	163	156	4.4
160	167	4.3	184	188	2.2
176	180	2.3	161	168	4.2
146	146	0	192	205	6.6
177	180	1.7	111	123	10.3

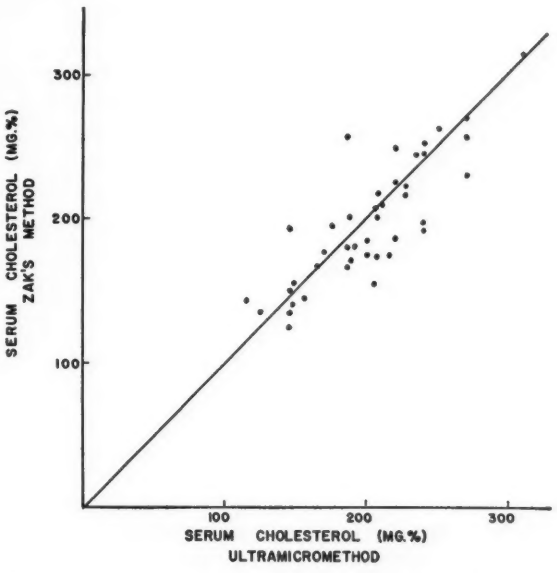

FIG. 3. Comparison of serum total cholesterol values obtained by the proposed ultramicromethod and the Zak technique.

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Low temperature chromatography of lipids on cellulose

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► The separation of neutral fat from phospholipids has long depended on their solubilities in acetone, and the term "acetone-insoluble lipids" is regarded as synonymous with phospholipids, although the separations obtained are not clear-cut without repeated precipitation. In recent years effective separations have been obtained using chromatography on silicic acid (1, 2), but it is possible that degradation of some of the phospholipids may take place (3, 4). Dialysis through a rubber membrane (5) has been used, but this is slow and is efficient only when required to remove small amounts of neutral fat from the phospholipid fraction (6).

Work in this laboratory (4, 7) has shown that phospholipids after dinitrophenylation and methylation can be divided into an acetone-soluble and an acetone-insoluble fraction. The phosphatidic acidlike phospholipids and those containing a dinitrophenyl group are acetone-soluble, while lecithin and the inositol phospholipids remain acetone-insoluble. The procedure described below has been found to effect a satisfactory separation of these two fractions.

The lipids are dissolved in petroleum ether, b.p. 40°-60°C (approximately 0.5 g. in 100 ml.), the mixture added to 90 g. of cellulose powder ("Whatman's for chromatography"®) and the solvent removed by heating for about 20 minutes at 60°-80°C in a stream of nitrogen to obtain a homogeneous mixture. A slurry of cellulose (30 g.) and acetone is poured into a chromatography column (5 cm. diameter by 35 cm.), the cellulose packed down with the aid of compressed air, and the level of acetone allowed to fall to within 1 cm. of the top of the cellulose. A stopcock at the bottom of the column is turned off and the column placed in a deep-freeze cabinet at −18°C, together with the mixture of lipid and cellulose, and acetone. When equilibrium is attained, the lipid-cellulose mixture is added to the column together with cold acetone, and air bubbles removed by stirring with a glass rod. The stopcock is now opened and 2 l. of cold acetone allowed to flow through the column; this quantity of acetone has been found adequate for complete elution of the acetone-soluble material. The column can then be removed from the deep-freeze cabinet and all the remaining lipids removed by elution with 2 to 3 l. of chloroform-ethanol (2:1, v/v).

It has been found that lower temperatures (−20° to −30°C) do not result in improved separations and that the ratio of acetone-insoluble material to cellulose is critical and should not be more than 1:400. It is also essential that the cellulose should be exhaustively extracted with chloroform before use to remove "lipids" amounting to about 0.1 per cent.

Table 1 gives the results of this separation as applied to dinitrophenylated and methylated phospholipids and it will be seen that the acetone-insoluble material

TABLE 1. SEPARATION OF DINITROPHENYLATED AND METHYLATED LIPIDS INTO ACETONE-SOLUBLE AND ACETONE-INSOLUBLE FRACTIONS

Rat Tissue	Total Weight	Acetone-Soluble		Acetone-Insoluble	
		P	DNP*/P	P	DNP*/P
	mg.	*μmole*		*μmole*	
Liver	587	255	0.57	175	0.0
Liver	954†	349	0.57	283	0.0
Heart	487	269	0.53	133	0.038
Kidney	334	125	0.59	75	0.02
Liver mitochondria	306	124	0.55	112	0.012
Liver microsomes	359	120	0.58	150	0.0

* Mole ratio.
† Excluding neutral fat.

contains little or no dinitrophenyl groups while, in the other fraction, the ratio of dinitrophenyl groups to phosphorus (DNP/P) was between 0.53 and 0.59. This fraction contains three main components: phosphatidylethanolamine and phosphatidylserine (DNP/g. atom.P = 1.0), "complex" amino-phospholipids (DNP/g.atom.P. = 0.5), and several components (some unidentified) with no DNP groups (7). Microbiological examination (8) showed that inositol was present only in the acetone-insoluble materials and that mild alkaline hydrolysis yielded glyceryl-phosphorylcholine from the acetone-insoluble materials but none from the acetone-soluble fraction.

The success in separating these phospholipid de-

rivatives prompted an investigation of the separation of neutral fat and phospholipids not dinitrophenylated or methylated. Table 2 shows that the acetone-soluble fraction contained little phosphorus. The percentage of phosphorus in the acetone-insoluble fractions corresponded to the values usually found for phospholipids, except in the case of the brain lipids where the values were consistent with the presence of cerebrosides in the proportions reported in the literature (9). The rat liver lipids, which had been labeled with P^{32} by intravenous injection of $NaH_2P^{32}O_4$ 90 minutes before the animal was killed, yielded acetone-soluble lipids containnig 0.21 per cent P with a specific radioactivity of 80 cpm. per μmole P and yielded acetone-insoluble lipids having an activity of 870 cpm. per μmole P. This demonstrates that the acetone-soluble phospholipids were not merely an adsorbed sample of the total phospholipids. It cannot be decided whether small quantities of acetone-soluble phospholipids were present in each sample or whether in some cases the separations were incomplete.

When lipids are treated with cold acetone, the precipitate contains entrained acetone-soluble compounds, while the supernatant contains acetone-insoluble materials which are solubilized in the high concentrations of neutral fat. Therefore, a clear-cut separation requires a complicated process of fractional precipitation and the smaller the sample, the more difficult it is to obtain a complete separation. It was believed that more efficient separation would be obtained if the lipids were spread out over a large area of cellulose (judged to be a suitable adsorbent) and then washed with cold acetone. The fact that the ratio of acetone-insoluble lipid to cellulose is critical demonstrates that adsorption occurs and that the process is not one of filtration.

In the present investigation the object has been to obtain two fractions only, but some indications have been observed that further fractionation of the acetone-soluble materials is possible. It may be practicable to effect other separations by varying the adsorbent, the solvents, and the temperature. Other fields in which this technique might be useful are the low temperature fractionation of fatty acids and the fractionation of triglycerides in studies of glyceride structure.

TABLE 2. SEPARATION OF NEUTRAL FAT AND PHOSPHOLIPIDS

	Acetone-Soluble			Acetone-Insoluble	
	Weight	P	Total Lipid P	Weight	P
	mg.	*per cent*	*per cent*	*mg.*	*per cent*
Sheep brain	210	0.0084	0.09	592	2.80*
Rat liver	186	0.21	3.3	321	3.55
Hen egg yolk	95	0.015	0.82	48	3.59
Rat brain	109	0.15	1.6	273	2.95
Rat heart	85	0.26	1.9	304	3.73

* Liebermann-Burchard test for sterols was negative.

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New Methods

► **New Methods is a listing of recent and pertinent references to analytical lipid methods published in other journals. It is compiled by H. A. Newman, Norman S. Radin, and David Kritchevsky. (References opened with No. 1 in the October, 1959, issue of the JOURNAL OF LIPID RESEARCH.)**

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Notice of Meeting

The Fifth International Congress on Nutrition will be held in Washington, D.C., September 1–7, 1960. For full information write to Dr. Milton O. Lee, General Secretary, 9650 Wisconsin Avenue, Washington 14, D.C.

Editorial Correction

Due to a printing error, several lines were incorrectly placed in the Summary to the Bradbeer and Stumpf article, page 214 of the April, 1960, issue of the JOURNAL. The entire Summary is therefore reprinted correctly below:

SUMMARY

A phosphatidic acid is the major lipid to become labeled when Pi^{32} (inorganic orthophosphate labeled with P^{32}), under conditions of oxidative phosphorylation, or ATP^{32} is fed to mitochondria from the cotyledons of germinating peanut seedlings. With ATP^{32} as the source of P^{32}, the only cofactor required was Mg^{++}. The stimulation of phosphatidic acid synthesis by an $\alpha\beta$-diglyceride provided support for the view that this synthesis is due to diglyceride phosphokinase activity in the mitochondria. Evidence is also presented that this enzyme preparation is capable of phosphorylating α-monoglycerides with the formation of monoacyl phosphatidic acids. When Pi^{32} was added to slices of peanut cotyledons, the phosphatidylcholine and phosphatidylethanolamine in the mitochondria obtained a much higher level of radioactivity than that observed in experiments with the isolated mitochondria.

INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4:** 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg.
gram	g.
milligram	mg.
microgram	μg.
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	$cm.^2$
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc.
microcurie	μc.
degrees of temperature	°C or °F

The expression mg.% should not be used, but should be written as mg. per 100 ml. For chemical nomenclature the JOURNAL will follow the conventions of *Chemical Abstracts*. The superscript ® should follow all trade names. When necessary for identification of other specific materials, use a footnote, including name and location of manufacturer.

References should appear in numerical order throughout the text. Bibliographic references must be typewritten, double-spaced, on a separate sheet, using the following style:

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Example: Garn, S. M., A. B. Lewis and D. L. Polacheck. *Science* **128:** 1510, 1958.

FOR BOOKS: Author's last name, initials. *Title of Book.* City, Publishing House, year, page number.

Example: Adolph, E. F. *Physiology of Man in the Desert.* New York, Interscience Publishers, Inc., 1947, p. 420.

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Example: Anker, H. S. In *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan, New York, Academic Press, Inc., 1957, vol. 4, p. 779.

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Mention of "unpublished experiments," "personal communications," etc., should be made as footnotes and not included in the references. References to papers which have been *accepted* for publication but not yet printed, are cited in the style as other references, followed by the words "in press."

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